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Anatomical and histological changes during the oestrous cycle in the mare

By John Hammond, F.R.S. and Kazimierz Wodzicki*

School of Agriculture, Cambridge and College of Agriculture, Warsaw

(*Received* 18 *November* 1940)

[Plates 1-4]

The mares chosen for the investigation of the changes in the reproductive organs during the oestrous cycle were kept under observation for some time before they were killed. The duration of the heat period in these animals was 7 days and the length of the dioestrus was 16 days. Ovulation takes place at about a day before the end of oestrus. The size of the ovary during the oestrous cycle is chiefly influenced by the growing Graafian follicle. The number of follicles present at different stages varies greatly. The numerous small follicles present at the beginning of oestrus disappear later in the cycle; it is suggested that this may be due to the lack of follicle-stimulating hormone.

The colour of the corpus luteum varies greatly at different stages of the cycle. The rupture of the follicle is associated with some bleeding. The active stage of the corpus luteum is very short, and the maximum diameter of the corpus luteum seems to be always below that of the Graafian follicle. The greater development of the Graafian follicle, with its secretion of oestrin, in the mare leads to its playing a more important role than in the cow and the sow, in which species the corpus luteum takes a more dominant part in the cycle. It appears that the much longer oestrus in the mare than in the cow is due to the longer time required by the follicle to come to the surface and to break through. This is probably due to the peculiar structure of the ovary in the mare, since the ovulation, which is spontaneous, can only occur in the small ovulation fossa. No pronounced secretion stage occurs during oestrus in the Fallopian tubes.

The anatomical and histological changes in the uterus of the mare during the cycle are slight as compared with those in species which have a specialized placentation, such as the rabbit, ferret and cat; they are similar to those changes which occur in a species with a simple placentation such as the pig.

Cyclic changes are described in the epithelial cells of the cervix, which acts as a large mucous gland. It is probable that ejaculation takes place directly through the cervix into the uterus.

The changes in the vagina proper and the vestibulum vaginae are described; they are less pronounced in the mare than in the cow, but some cornification and leucocytosis, similar to that found in some rodents, occurs.

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INTRODUCTION

The study of the reproductive processes in the mare is of interest from several points of view. On the one hand we are interested in the improvement of the low fertility in the mare, which in Great Britain varies from 53 to 59 % in different breeds. On the other hand we are also interested in these processes from a theoretical standpoint, in view of the fact that the mare is an animal with a primitive placentation. As was shown by one of us (Hammond 1927) for the cow there exists a correlation between some of the anatomical and histological changes in the reproductive organs and the physiological processes during the oestrous cycle. In the present paper an attempt is made to point out these changes in some reproductive organs of the mare during the oestrous cycle.

MATERIAL AND METHODS

The chief difficulty in such studies is that the mare is an expensive animal to keep and to kill, so that the number of animals used for experiments must necessarily be limited. At the Animal Research Station, School of Agriculture, University of Cambridge, a uniform set of six Welsh pony mares from the same strain from one mountain were kept in a separate paddock during the winter of 1932 and the spring of 1933. Every day the mares were tested by a vasectomized stallion of the same breed in order to find out the length of their oestrous cycles and the intervals between them. In this way it was considered that the actual time the experimental mares came on or went off heat could be determined within one day. As has been shown by Hammond (1934, 1938*a*, *b*) and also by Day (1939*a*, 1940), the length of the heat period varies during the year from 54 to 3 days, although the interval between the termination of the last heat period and the beginning of a new one is generally the same—16–17 days. By testing with vasectomized stallions it was found that the duration of oestrus just before killing was about 6–7 days. The time of killing and period of the oestrous cycle during which the experimental mares were killed are given below (table 1).

Immediately after killing, the animals were dissected and the parts of the genital tract were examined in fresh state and weighed. Most of the parts were then fixed in 10 % formalin solution and afterwards stained mostly in Delafield's haematoxylin or Haemalum of Mayer or iron haematoxylin and van Gieson. Some parts, such as pieces of the cervix, upper vagina, etc., were fixed in mercuric chloride and then stained with Mayer's mucicarmine, counterstaining with iron haematoxylin. For histological study the following parts were fixed separately: ovarian part of tube, horn of uterus, cervix, upper vagina and vagina below urethra.

TABLE 1

No. of mare ...	C_4	C_{11}	C_9
Date of killing	9 June	9 June	20 June
Stage of oestrous cycle at killing	2 days before heat	1st day of heat	5th day of heat
Remarks	—	—	—

No. of mare ...	C_8	C_7	C_6
Date of killing	26 June	7 June	9 June
Stage of oestrous cycle at killing	6th day of heat	2 days after end of heat	8 days after end of heat
Remarks	Foaled, 19 April 1933	—	—

1. ANATOMICAL CHANGES IN THE OVARIES

General remarks

A detailed description of the ovary in the mare is given by Williams (1931), Sisson (1930) and Schmaltz (1921*a*, *b*), so that it will only be necessary to make a few general statements, and then to describe the variations that take place in this organ during different stages of the oestrous cycle.

As is well known the ovary of the mare is bean-shaped and is the largest of all the domestic animals, being *ca.* 5½–8 cm. long and 2½–4 cm. thick, with a weight of *ca.* 40–70 g. The ovaries are normally larger in young animals than in adult ones: the gland attains its maximum size when the animal has reached the age of 3 or 4 years. They vary greatly in size and form according to the presence or absence of Graafian follicles or a corpus luteum. When these are absent there is in the free border a well-marked narrow depression up to 7 mm. deep, the 'ovulation fossa'. The peculiarities of the ovary in the adult mare lie not only in their relatively large size and shape, but also in that, unlike the ovary of other species, it does not consist of a cortex (zona parenchymatosa) in which the follicles are situated and a medulla (zona vasculosa) which contains the vessels and nerves. Instead the serosa covers the whole ovary with the exception of the 'ovulation fossa'. The latter is covered by a layer of short polygonal cells, a remnant of the primitive germinal epithelium. In the adult animal the follicles are distributed throughout the interior of the ovary but rupture only in the ovulation groove and not on the general surface of the ovary. Kupfer (1928) found a striking similarity between the ovarian changes in the donkey and the mare. He has reported that in young donkeys mature follicles may appear at different points of the ovary, and that the rupture of follicles is by no means confined to the 'ovulation fossa'. In the mare's ovary the larger blood vessels are distributed mostly superficially over the general surface of the ovary just under the fibrous capsule. In a functional ovary, just under the capsule, there is a more or less thick layer of interstitial cells lying in the stroma: these can easily be picked out by their colour which varies from brownish orange to brownish yellow. The middle of the ovary is generally occupied by the stroma of the ovary, a network of connective tissue in which are developing follicles, and corpora lutea, the newest ones being pear-shaped with the thinner part pointing towards the ovulation fossa.

The developmental changes which bridge the gap between the structure of the ovary of the mare and those of other domestic species have been described by Petten (1932), Cole, Howell and Hart (1931), and Cole, Hart, Lyons and Catchpole (1933).

Size of ovaries in different stages of the oestrous cycle

The changes occurring in the ovaries during different stages of the oestrous cycle have been studied by first observing and weighing the ovaries in a fresh condition and, later, after fixation by making moderately thin sections, to cut through the largest possible diameter of the corpus luteum or largest follicle in the ovary; as was done by Hammond (1927) for the cow, measurements of the maximum cross diameters of these have been taken by the use of dividers and a scale. The rupturing of only one follicle permits the study of the relationships in size between the different parts of the ovary during the oestrous cycle.

A study of the weights of each ovary at different stages of the cycle shows (see tables 2 and 3) that there are large differences between the weights of the two ovaries of the same animal at different stages of the cycle. Before oestrus the relative weight of one ovary to the other is about 3:1 but by the 5th day of heat this relation is about 6:1. It can be easily seen that this difference is very largely influenced by the growth of the new Graafian follicle, and less by the presence of a new corpus luteum. These changes become more characteristic and evident if the weight of the inactive ovary is deducted from the weight of the one with the follicle and new corpus luteum (active ovary, table 2). The curve in figure 2, in which these weights are plotted on each day of the oestrous cycle, shows that the increase of weight in the ovaries is due in the first place to the growing Graafian follicle between 2 days before heat and especially during the first few days of oestrus: the largest weight of the ovary in our experimental mares occurs on the 5th day of oestrus, just before ovulation (see figure 1, plate 1). The weight of the ovary on the 6th day of heat is almost as small as that 2 days before heat; this is due, as will be shown below, to the rupture of the follicle and loss of the liquor folliculi before the formation of a new corpus luteum. The increase of weight between the 6th day of oestrus and the 2nd day after end of heat coincides with the formation of a new corpus luteum, and the ovarian weight then begins to decrease till the 8th day, at which time it is rather smaller than at the 2nd day.

Concluding, it can be said that, in the mare, the size of the ovary is mostly influenced by the growing follicle and slightly only by the presence of a new corpus luteum. This is the contrary to what was found by one of us (Hammond 1927) in the cow as also by Corner (1921) in the sow, where the weights of ovaries are very largely influenced by the presence of corpora lutea and less by the growing follicles.

The development of the Graafian follicles

The development of the Graafian follicles was studied by Kupfer (1928) in the donkey. He has found that only one of the many follicles which appear macroscopically on the active ovary reaches full maturity and ruptures; the remainder of the follicles are reduced and appear as atretic follicles or remain over for a new interovulation period. As is well known in the mare only one follicle usually ripens at each period.

As is shown in tables 2 and 3, in figure 1, plate 1 and figure 3, the development of the Graafian follicle is peculiar in the mare and differs in many points from the development of the follicle in the cow, as well as in some other domestic animals. The observed follicles were divided in four groups according to size, the largest of which would be due to ripen at next oestrus, while the smallest consisted of very small follicles only.

The numbers of follicles in both ovaries vary greatly in different stages of the oestrous cycle (tables 2 and 3): 2 days before heat there are only four follicles in both ovaries, whereas 2 days afterwards on the 1st day of oestrus there are twenty-two follicles, mostly very small ones. The same thing is found on the 5th day of heat, when there are a similar number of twenty-one follicles. On the 6th day of heat, or just after ovulation, the number decreases to seven relatively small follicles. In the period just

(2 days) after the end of heat the smallest number of follicles, one in each ovary, is to be observed. At 8 days after the end of heat there are eight follicles. Of course the number of observations are too few on which to base general conclusions, but it seems to be certain that there is a relation between the number of growing new small follicles and the presence of one large Graafian follicle during the beginning of the period of oestrus. Afterwards these small follicles usually degenerate under the influence of the developing new corpus luteum or absence of the follicle-stimulating hormone from the anterior pituitary.

Tables 2, 3 and 4 give a bird's-eye view of the number and average diameters of the follicles and corpora lutea as well as a description of some particulars, in both ovaries, of our experimental mares in different periods of the oestrous cycle.

The growth of the follicles which are to ripen at the next oestrus is of interest. As is shown in figure 3, the enlargement of the follicle that is to ripen begins just after the end of oestrus, and takes place gradually, due to the influence of the corpus luteum of the last heat period. A sudden enlargement takes place just before the beginning of the new heat period, and a slightly smaller enlargement takes place between the 1st and 5th day of oestrus. The second follicle follows the first one, decreasing in size after ovulation has taken place, when a new corpus luteum is formed.

Ovulation

Ovulation occurs spontaneously in the mare, as in many other domestic animals. The exact time of the ovulation is from the breeding point of view a very important matter. Other observations, e.g. artificial rupturing of the follicle on the 2nd and on the 4th days of heat (Hammond 1938*a*; Day 1939 *a*, *b*), point to the fact that the shedding of the egg occurs about 24 hours before the end of heat. This is confirmed by our findings: the rupture of the Graafian follicle occurs on the 6th day of oestrus (figure 1, plate 1 and figures 2, 3). Just before this, on the 5th day of oestrus, the liquor folliculi becomes much more fluid and, when fixed in formalin, is not so firm as in the smaller ones, which have not yet ripened.

The corpus luteum

The changes which occur in the corpus luteum are very interesting (tables 2 and 4, figures 1, 2, 3).

The new-formed corpus luteum appears on the 6th day of heat, ovulation occurring, as was mentioned above, between the 5th and 6th day of oestrus.

TABLE 2. PARTICULARS OF OVARIES OF MARES KILLED AT DIFFERENT STAGES OF THE OESTROUS CYCLE

Stage of oestrous cycle	2 days before heat	1st day of heat	5th day of heat		6th day of heat	2 days after end of heat	8 days after end of heat
No. of mare	C_4	C_{11}	C_9		C_8	C_7	C_6
Weight of ovaries (in g.):							
Active ovary (with the follicle before ovulation or with a new corpus luteum)	15·63	29·72	62·15		25·42	27·22	20·10
Inactive ovary (with old corpus luteum)	5·28	10·05	9·72		12·80	5·93	11·44
Weight of active ovary minus that of inactive one (approx. weight of follicle)	10·35	19·67	52·43		12·62	21·29	8·66
Size of corpora lutea (diam. in mm.):							
Of new generation	—	—	—		25·5	30·5	20·0
Of last generation	17·0	15·0	9·0	Ovulation	11·0	8·0	8·5
Of previous generation	—	3·0	5·0		5·0	—	2·0
Size of follicles (diam. in mm.):							
Large to ripen at next oestrus	19·0	33·5	42·0		11·0	12·0	22·0
Medium	13·5	11·0	16·0		9·0	10·5	19·5
Small	6·0	9·0	14·5		7·0	—	13·5
No. of all follicles	4	22	21		7	2	8
Colour of corpus luteum:							
Of new generation	—	—	—		Pale	Pale with chocolate blood spots	Buff-orange
Of last generation	Buff	Buff-orange	Brownish-orange		Yellow-orange	Reddish orange	Orange scarlet
Of previous generation	—	Scarlet-orange	Brownish orange		Dark orange	—	Orange

TABLE 3. THE NUMBER AND AVERAGE DIAMETER (MM.) OF THE FOLLICLES IN DIFFERENT STAGES OF THE OESTROUS CYCLE

Stage of oestrous cycle	No. of mare	Total no. of follicles	Largest follicles to ripen at next oestrus		Moderately large follicles		Small follicles		Very small follicles	
			No.	Average diam.	No.	Average diam.	No.	Average diam.	No.	Average diam.
2 days before heat	C_4	4	1	19·0	1	13·5	1	6·0	1	4·0
1st day of heat	C_{11}	22	1	33·5	1	11·0	1	9·0	19	9 (4·0– 9·0) 10 (1·5– 4·0)
5th day of heat	C_9	21	1	42·0	1	16·0	1	14·5	18	12 (4·0–11·0). 6 (2·0– 4·0)
6th day of heat	C_8	7	1	11·0	1	9·0	1	7·0	4	2 (4·0– 5·0) 2 (3·0– 4·0)
2 days after end of heat	C_7	2	1	12·0	1	10·5	1	—	...	—
8 days after end of heat	C_6	8	1	22·0	1	19·5	1	13·5	5	3 (4·0–10·5) 2 (2·0– 4·0)
Average diam. of the follicles	—	—		11·0–42·0		9·0–19·5		6·0–14·5		1·5–11·0

The pale colour is evidently due to the absence at this stage of lipoids in the luteal cells. The large, new corpus luteum (29–24 mm. diameter) consists of the folded walls of the follicle with numerous blood vessels and a central cavity (9 × 16 mm.). This cavity is continuous with the large point of rupture of the follicle, which has not yet been occluded by growth, and in the middle contains a small amount of secondary liquor folliculi.

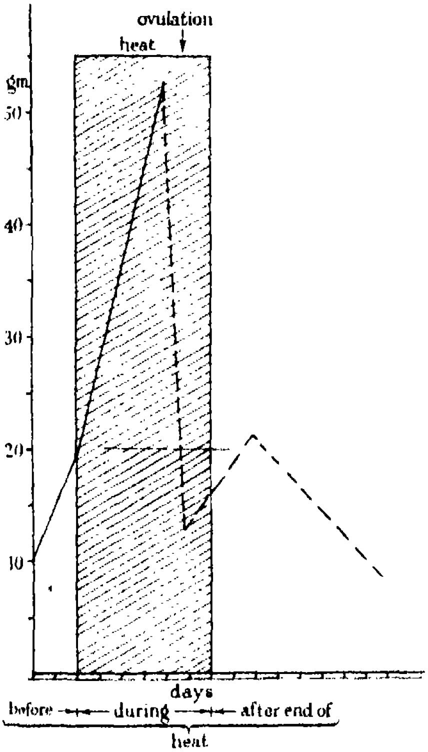

FIGURE 2. Curve showing the weight of the active ovary minus the weight of the inactive, during the oestrous cycle. Continuous line—ovaries with growing Graafian follicle; broken line—ovaries with active corpus luteum.

It is evident from comparison with the changes which take place in the formation of the corpus of the rabbit (Hammond and Marshall 1925) and of the cat (Liche 1939, and unpublished data) that at this stage the corpus luteum has only just been formed.

In the next stage, 2 days after the end of heat, the corpus luteum itself is also pale, but it is suffused with chocolate blood spots. The large red point of ovulation in the fossa is still visible. At this stage it has the largest dimensions (25 × 29 mm. diameter) at any time during the oestrous cycle of the mare. Evidently this is the period of the development of the structure of the corpus luteum. The ingrowing cells of the walls are pale, the point of rupture is occluded, and a considerable amount of secondary liquor folliculi has accumulated in the centre; this is of chocolate colour and is caused by the rupture of blood vessels at the time of ovulation. This coagulum of blood in the centre of the corpus luteum has been described in the donkey by Kupfer (1928), and may be seen in the centre of the corpus luteum long after rupture.

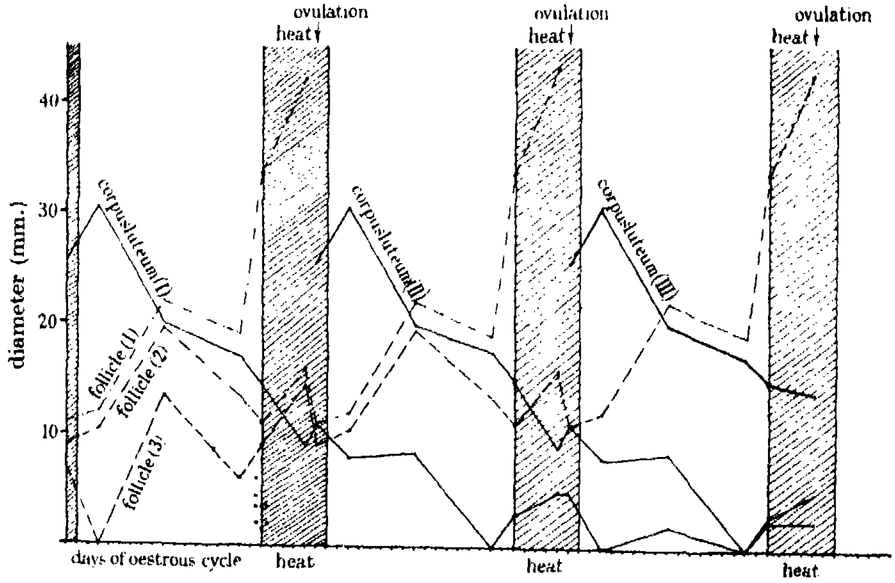

FIGURE 3. Curves of growth of follicle and corpus luteum during the oestrous cycle.

In the next period, between the 2nd and 8th day after the end of heat, the structure of the corpus luteum is completed but its size becomes reduced, and it now has a smaller diameter than the largest of the newly forming Graafian follicles (tables 2 and 3). The consistency is firm with occasional blood vessels in the middle, and is of a buff-orange colour.

In the next stages (2 days before and during the next oestrus) the diameter of the corpus luteum is still further reduced, and its colour changes from buff-orange to reddish and then to scarlet or brownish orange.

It is, of course, difficult to state very accurately the course of these changes in our very limited number of observations, as one does not know how much may be due to individual variability: but, as stated above, the mares selected for this purpose were as uniform as it was possible to obtain them. It can be stated definitely, however, that a corpus luteum of a previous generation can exist, although much reduced in size, side by side with one of the last generation and a third one from the new generation (table 2 and figure 3).

In conclusion, it is evident that, in the mare, as compared with the cow, the active stage of the corpus luteum is very short, in spite of its persistence in a relatively inactive state during two or three oestrous cycles. It would seem (see figure 3) that the follicle takes a much more prominent part in the cycle than it does in the cow (see Hammond 1927, diagram 1). That is, in the cow the corpus luteum, with its secretion of progesterin, plays a much larger part in the cycle than in the mare, while in the latter the follicle with its secretion of oestrin is a much more prominent feature of the cycle. It seems possible that this, and the long duration (6–7 days) of oestrus, may be associated with the peculiar structure of the ovary in the mare, as compared with other species, for as ovulation can only take place in the ovulation fossa, it takes longer for the growing follicle to come to this surface and break through them than if it could come to the surface directly above where it started to develop.

II. THE ANATOMICAL AND HISTOLOGICAL CHANGES IN OTHER PARTS OF THE GENITAL TRACT OF THE MARE

In the subsequent pages will be given a short description of the changes observed in tubes, uterus, cervix and upper and lower vagina of the experimental mares during different stages of the oestrous cycle.

(*a*) *The Fallopian tubes*

The Fallopian tubes in the mare are 30–70 mm. long, and consist of a proximally situated ampulla tubae with a diameter of 4–8 mm. and a uterine end with a diameter of only 2–3 mm. The muscular coat of the oviducts is characterized by its density, which gives to it a firm consistency, and its paleness.

The tubes were studied in sections from the same part of the ampulla tubae in each case.

The tube has the same layers of tissues as the uterus: (1) tunica serosa, (2) muscularis of a weaker longitudinal and thicker circular smooth muscle layer, and (3) mucosa. The most distinctive feature of the tube in the mare is, according to Schmaltz (1921*b*), the large number of primary and secondary folds (up to sixty); this could be observed on our material. The mucosa consists of a ciliated epithelium and a thin connective tissue with small blood and lymphatic vessels. Some authors (von Ebner 1902; Kuhn 1906) have also found some non-ciliated epithelium cells of narrow size and a longitudinal nucleus. Courrier (1930) and others have found in dogs, pigs, bats and guinea-pigs, characteristic changes in which during oestrus the cilia are lost and a mucous substance is produced by these cells and excreted into the lumen.

The fixation of our material in formalin does not allow a very accurate observation of the changes in this part of the genital tract during the different stages of the oestrous cycle. The observed differences are very slight as can be seen in table 5.

Some differences can be seen in subepithelial capillaries which are very small 2 days before heat. In the beginning of the heat period they are a little enlarged, and towards the end of oestrus are more prominent: 2 days after the end of heat they become quite small again.

The changes in the epithelium of tubes are also very slight: 2 days before heat as well as 8 days after the end of heat the cells are cubical in size. At the beginning of oestrus they become larger in size and of columnar shape, with more leucocytes in the stroma. On the 5th day of oestrus the epithelium cells seems to be swollen, perhaps partly filled with secretion, and contain many leucocytes in the stroma, especially when compared with the stage of 8 days after the end of heat. Between the 5th and 6th day of heat there are only slight differences. The 2nd day after the end of oestrus the epithelium cells appeared to be again more cubical in shape.

To summarize, the histological changes in the tube of the mare are relatively slight and there apparently exists in the mare no very pronounced secretion stage of the Fallopian tubes, such as has been described by Courrier (1930) in other animals.

(*b*) *The uterus*

The uterus of the mare consists of two separate horns joining together distally to form the body of the uterus. It constitutes a somewhat cruciform organ, the horns leaving the body laterally at right angles or slightly recurved. The parts stained for histological study were always taken from approximately the same region of the horn half way between the body and the end of the Fallopian tubes.

Anatomical changes. Just after killing, the uterus together with the cervix of each mare was weighed and observed in fresh state. As shown in table 4 there was no correlation between the weight of these parts and the stage of the oestrous period: the larger weights of C_8 and C_4 are due to the fact that these mares were pregnant during the previous season.

The changes in the colour of the folds of the mucosa and in the general appearance of the uterus were more pronounced and interesting. Table 4 gives these exactly as they were recorded in our dissection book. The observed changes of colour from brownish yellow to pinkish yellow are undoubtedly connected with the congestion of the blood vessels which occur during the oestrous cycle.

The turgid condition of the uterine folds of the mucosa just before and at the beginning of heat appear to be followed by a more flaccid condition towards the end of heat and in the following days.

Histological changes. We shall begin by giving a bird's-eye view of the chief particulars of the histological structure of the uterus of the mare as described by the authors quoted above, and then point out some characteristics of the uteri of the experimental mares.

The general division of the uterine walls into serosa, muscularis and mucosa applies to the uterus of the mare; but the muscularis is not accurately separated into three layers: the muscle fibres from the weaker muscularis longitudinalis go simultaneously into the vascularis, and it is very difficult to separate the last one from the muscularis circularis.

The mucosa consists of three layers: (1) tunica propria, (2) uterine glands, and (3) stratum epitheliale. The first consists of extraordinarily loose connective tissue with a deeper glandular layer. According to Schmaltz (1921*b*) there are three layers as follows: (*a*) tunica propria, (*b*) stratum reticulare, and (*c*) stratum fibrillare.

The uterine glands are more numerous in the horns than elsewhere; they are long and have a tubular shape. As can be observed in the transverse sections they are long tubes which have a similar diameter in both the superficial and deeper layers. The tunica propria is peculiar in the mare in that it consists of loose connective tissue which, according to some authors, contains large lymphatic spaces. The epithelium of the glands has one single layer of columnar cells like those of the stratum epitheliale.

The stratum epitheliale is, according to Schmaltz (1921*b*), formed by high, but not ciliated epithelial cells in one layer with longitudinal nuclei situated on the base of the cells. The mucosa is formed by a loose connective tissue. From place to place there are large, pigmented, ovoid or round cells with a well-marked nucleus and vacuoles. The layer of glands in the horns is about 1 mm. in depth.

In our material we could confirm the above particulars of the structure with the addition of the observations shown in table 5 and figures 4–9 (plate 2). The extreme microscopical changes which occur during the cycle are seen between the stage of 2 days before heat (C_4) and the stage of the 2nd day after the end of heat (C_7). The superficial layers of cells on the 2nd day after the end of heat are vacuolized and the tissues become less dense than before heat begins. This takes place in connexion with the changes in the permeability of the walls of the blood vessels and the increasing of fluids during oestrus, such as also occurs during pregnancy. In C_4 (2 days before heat) there are fewer layers of cells in the epithelium than in C_7 (2 days after heat), in which the epithelium cells are higher, while lymphocytes can be seen passing through them. The changes in the size of the lumen of the superficial glands are small, but 2 days after the end of heat (C_7) they are wide, but become smaller again 8 days after (C_6). The stages of proper oestrus (C_{11}, C_9 and C_8) which are marked by a widening of the lumen of the uterine glands are rather like the appearance in the 3rd month of pregnancy, although not so pronounced.

The surface of the mucosa varies, too, in the different stages, as can be seen in figures 4–9 (plate 2). In the stages of 2 days before and the 1st day of heat the small surface folds of the uterine mucosa are relatively wide. On the 5th and 6th days of heat the surface of the mucosa becomes flattened with practically no folds, while after the end of heat (C_7 and C_6) the surface folds become marked again.

Taken as a whole these cyclic changes in the uterine mucosa of the mare are not nearly so marked as in such species as the cow, sheep, rabbit and ferret, but are more nearly similar to those which occur in the sow (Corner 1921). That is, the changes occurring in the uterine mucosa during the cycle are far less marked in those species which have a simple placenta than in those which have a more specialized placentation.

(c) *The cervix*

The cervix or neck of the uterus is, according to Schmaltz (1921*b*) and Williams (1931), a part of the pars indivisa uteri, and has no well-marked canalis cervicis, thus differing from the cow. The cervix rather protrudes into the upper vagina with a well-marked orificium externum. The lower part, which is about half of the total length of 6·5 cm., is distinguished by the presence of large folds (plicae palmatae). The cervix consists of a powerful, sphincter-like segment of the genital tract, which serves to separate, anatomically and physiologically, the uterus from the vagina.

Histologically there is a mucosa and a muscularis. The former is without the characteristic uterine glands, but has a well-developed subepithelial blood-vessel layer and, as mentioned above, well-marked primary and secondary folds. The whole mucosa produces as a large gland the most characteristic feature of this part of the genital tract—the mucus. The muscular layer in the cervix is well developed, thicker than in the uterus (10–20 mm.) but, according to Schmaltz (1921*b*), it is not so compact on account of connective tissue.

The mucous secretion and the degree of opening of the lumen of the cervix in the oestrous cycle were observed by Day (1939*a*): "In dioestrus, the cervix is tightly constricted and erect, a pale pink in colour, and the mucin is scanty and rather tenacious to the touch. In oestrus there is a gradual increase in the vascularity of the vagina and cervix, the mucin is more plentiful and is quite fluid and slippery to the touch." Furthermore, he observed a complete relaxation of the cervix during oestrus, so that it lay on the floor of the vagina and appeared somewhat oedematous.

Anatomical changes. The observed anatomical changes are recorded in table 4. The cervix was of pale appearance before (C_4) and on the 1st day of heat (C_{11}), and was congested from the 5th day (C_9) until the end of heat. The small quantity of rather sticky mucus observed before and in the beginning of oestrus considerably increases towards the end of heat, and becomes of thinner consistency. After heat (C_7) it again becomes moderately thick and decreases in quantity.

Not without interest are our observations concerning the degree of opening of the lumen of the cervix (table 4). On the 5th and 6th day of heat, i.e. in the second part of oestrus, as well as shortly after the end of heat (C_7), the muscles are relaxed and the aperture of the cervix is fairly wide open. Later, in the stage of 8 days after the end of heat (C_6), the lumen becomes very small again.

In our mare C_8 (6th day of heat), killed immediately after service, we observed the presence of a lot of sperm in the cervix; these, however, were not motile. It may be concluded that, at least in this case, the stallion ejaculated through the cervix. The presence of non-motile spermatozoa may be explained by the consistency of the mucus in the cervix; the sperm in the uterus were motile.

Histological changes. The histological examination confirmed the anatomical particulars described above. As stated, apart from the adventitia and muscularis, there is in the cervix a mucosa, thrown into a large number of primary and secondary folds. The mucosa has a core of connective tissue with finer blood vessels and is covered by an epithelium, which forms simple sacculated glands. The shape of these, especially in the secondary folds, is swollen and also the number of folds seems to increase at the end of heat and shortly afterwards.

The character of the epithelium varies considerably in different periods of the oestrous cycle (table 5). At the beginning of heat (C_{11}) the epithelial cells slightly lengthen, and by the 5th and 6th days (C_9 and C_8) they are nearing their maximum: 8 days after the end of heat (C_6) they have almost returned to their cubical form.

An explanation of these changes is found in preparations stained for mucin (figures 10–13, plate 1). Thus one can observe an enlargement of the mucosa, as shown by its greater thickness. Its cells are swollen during the second period of the oestrous cycle and the shape and number of secondary folds is increased. The enlargement that takes place during oestrus is due to an accumulation of mucus in the outer edges of cells, a varying amount being set free at the base of the folds. Two days before heat (C_4) the epithelium cells are mostly cubical with a few cells containing mucus; there is a very thin layer of mucus outside the cells in the vaginal lumen. On the 1st day of heat (C_{11}) they lengthen and are partly filled with mucus, the mucous layer lying outside the cells becoming more continuous; many leucocytes are to be seen in the submucosa, and some of them wander through the epithelium. Finally the connective tissue also loses its compact appearance during the first days of heat.

The 5th day of heat presents the following changes: the loose connective tissue is in some places filled with many leucocytes, and the blood vessels are enlarged and partly congested. The epithelium cells are swollen and rather columnar in shape; they are partly filled with mucus, which forms a wide layer outside the cells in the lumen of the cervix. As was to be expected, the next stage (the last day of heat, C_8) presents great changes. Even if the extravasation and congestion of blood vessels is partly due to a foregoing coitus, the climax of these processes is shown by the swollen shape of the epithelium cells, the mucus which fills them and, lastly, by a quantity of mucus in the lumen.

In the stages which follow the end of heat there is a gradual return to normal: 2 days after the end of heat the shape of the epithelial cells becomes more cubical, and there is a small amount of mucus outside the cells; a larger quantity still fills them. Finally, 8 days after the end of heat (C_6), the blood vessels in the mucosa are just visible, together with a few leucocytes, and the connective tissue is again more compact. In this period the mucus is produced by only certain cells, others having very little mucus in their body.

Taken as a whole, the changes in the epithelium of the cervix of the mare during the oestrous cycle, although not so pronounced as in the cow (Hammond 1927), are in principle the same: the liquefaction of thick

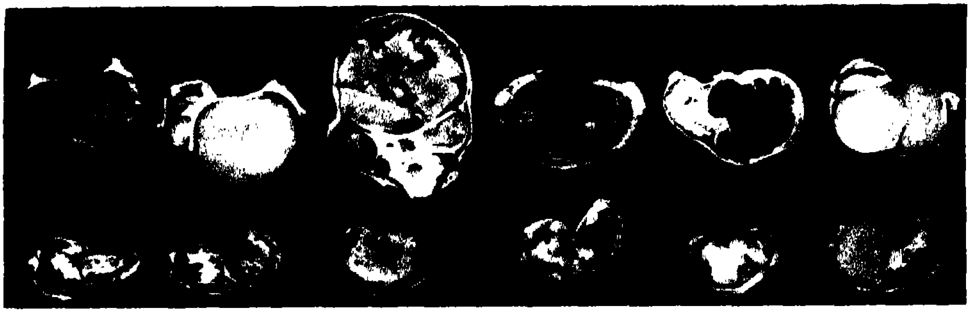

Figure 1. Sections of ovaries of the experimental mares at different stages of oestrous cycle. *Right and left ovary are shown one above the other. On the top row the* Graafian follicle is seen ripening. It enlarges greatly during the heat period and *ruptures between the 5th and 6th day of oestrus; from the walls of the ruptured* follicle a corpus luteum is formed, at first dark from the blood and secondary liquor folliculi, which breaks into the follicle. For other particulars see tables 2–4 and text.

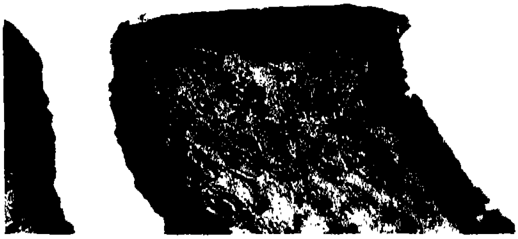

Figure 10

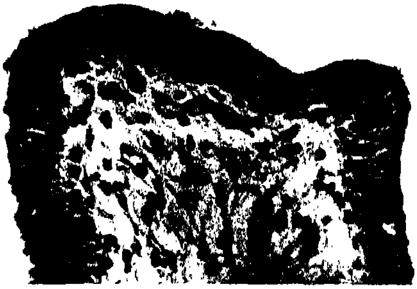

Figure 11

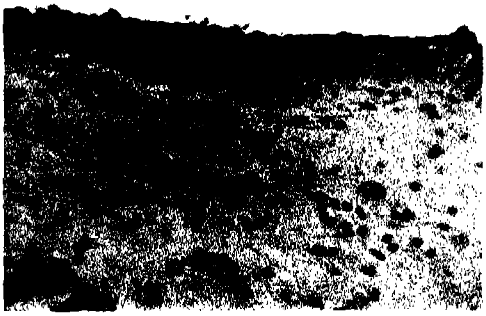

Figure 12

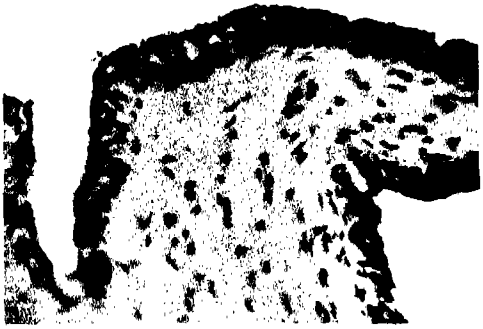

Figure 13

Figures 10–13. Sections through the cervix mucosa of the experimental mares. About × 250. Stain: Haemalum-Mayer's mucicarmine. Figure 10, C_4, 2 days before heat; figure 11, C_8, 5th day of heat; figure 12, C_8, 6th day of heat, and figure 13, C_6, 8 days after end of heat. Note the layer of mucin (stained dark) on the outer layer of the epithelium.

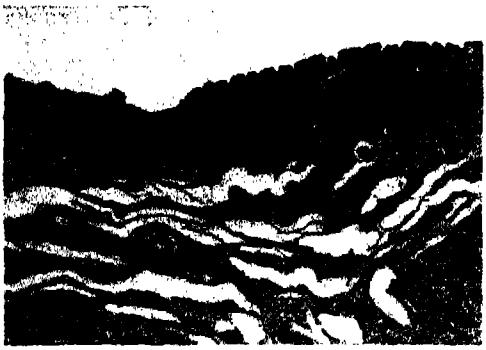

Figure 4

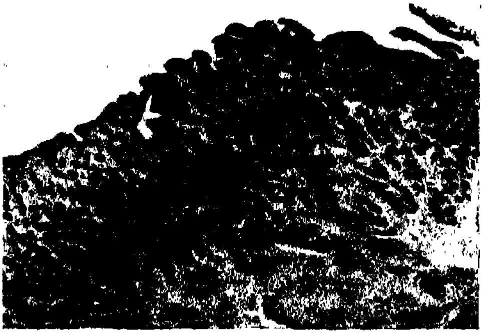

Figure 5

Figure 6

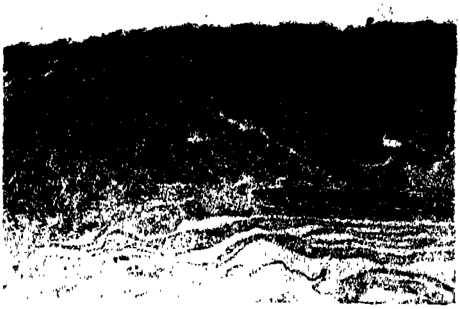

Figure 7

Figure 8

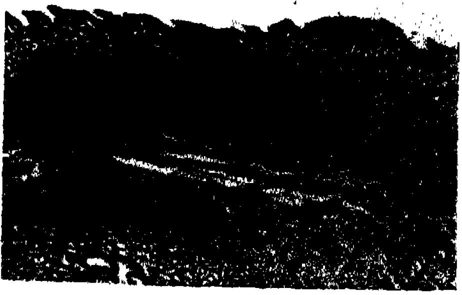

Figure 9

Figures 4–9. Sections through the uterine mucosa of the experimental mares. × 35. Figure 4, C_4, 2 days before heat; figure 5, C_{11}, 1st day of heat; figure 6, C_9, 5th day of heat; figure 7, C_8, 6th day of heat; figure 8, C_7, 2 days after end of heat, and figure 9, C_6, 8 days after end of heat.

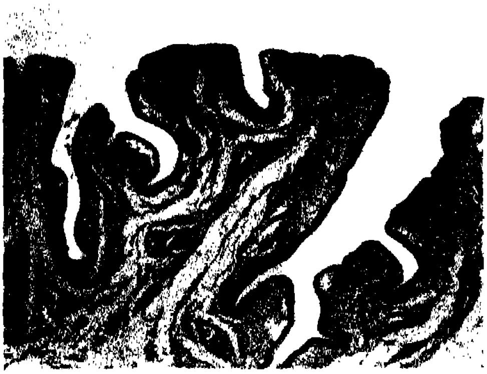

FIGURE 14

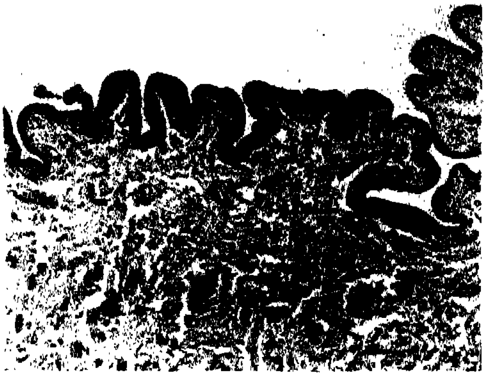

FIGURE 15

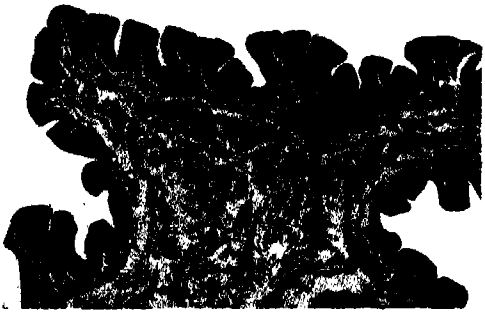

FIGURE 16

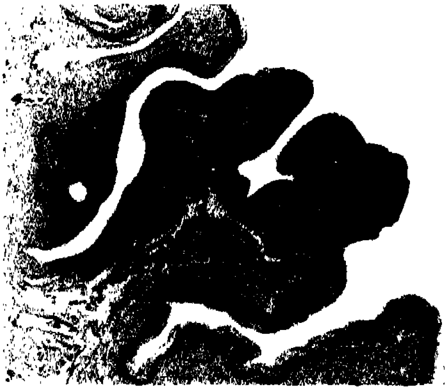

FIGURE 17

FIGURE 18

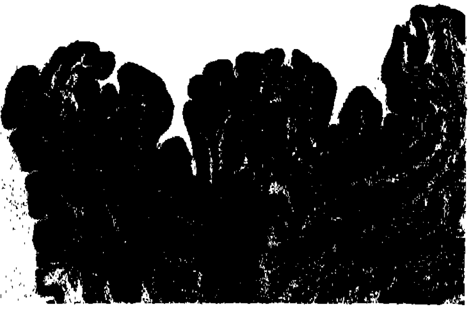

FIGURE 19

FIGURES 14–19. Sections through the upper vaginal mucosa of the experimental mares. About × 40. Figure 14, C_4, 2 days before heat; figure 15, C_{11}, 1st day of heat; figure 15, C_9, 5th day of heat; figure 17, C_8, 6th day of oestrus; figure 18, C_7, 2 days after end of heat, and figure 19, C_6, 8 days after end of heat.

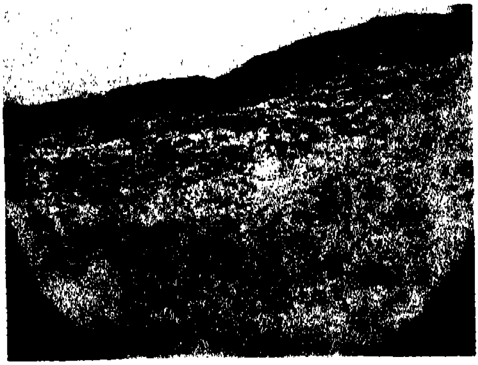

FIGURE 20

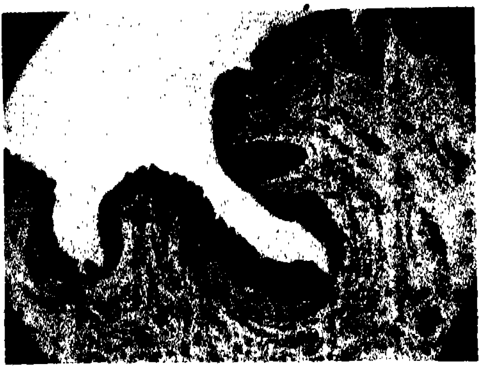

FIGURE 21

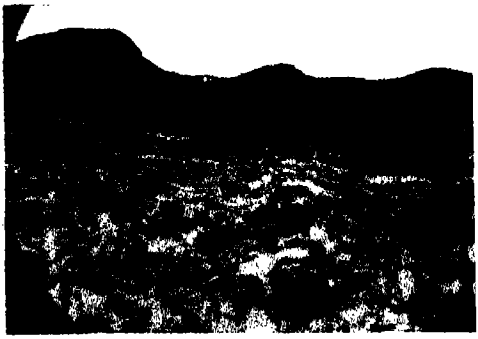

FIGURE 22

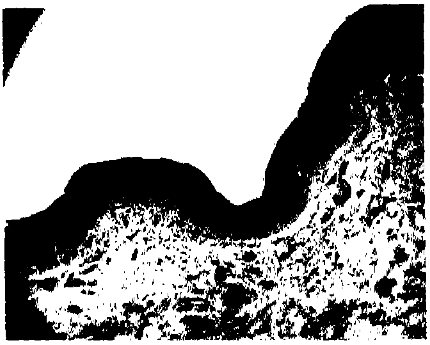

FIGURE 23 A

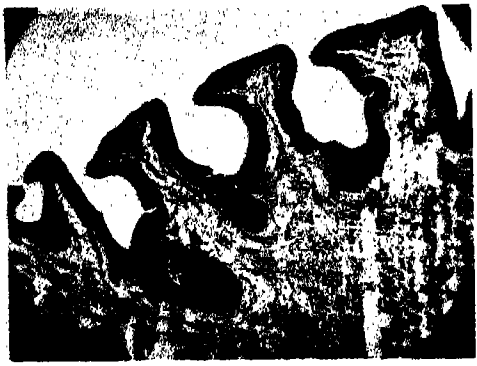

FIGURE 23 B

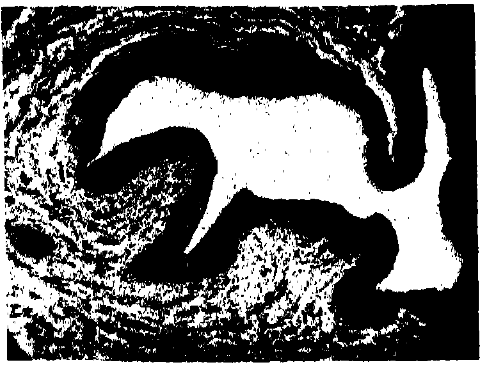

FIGURE 23 C

FIGURES 20–23. Sections through the vestibulum vaginae mucosa of the experimental mares. About ×40. Figure 20, C_9, 5th day of oestrus; figure 21, C_8, 6th day of oestrus; figure 22, C_7, 2 days after end of heat, and figure 23, C_6, 8 days after end of heat.

TABLE 4. PARTICULARS OF OVARIES, UTERUS, CERVIX, VAGINA AND UDDER OF MARES KILLED AT DIFFERENT STAGES OF THE OESTROUS CYCLE

Stage of oestrous cycle	2 days before heat		1st day of heat		5th day of heat			6th day of heat		2 days after end of heat		8 days after end of heat	
No. of mare	C_4		C_{11}		C_9			C_8		C_7		C_6	
Ovaries:	Left ovary	Right ovary	Left ovary	Right ovary	Left ovary	Right ovary		Left ovary	Right ovary	Left ovary	Right ovary	Left ovary	Right ovary
Weight (in g.) and size	5·28, Small	15·63. moderately large	29·72, large	10·05, small	62·15, very large	9·72, small		12·80, small	25·42, large	27·22, large	5·93, small	20·10, moderately large	11·44, small
Follicle	—	Two moderately large follicles, protruding *from* the surface of the ovary	Very large follicle, thin at surface of ovulation fossa	Small follicle	Very large	Two small follicles		No follicle visible	Just ovulated, flabby, congested with fresh blood on the surface and under the surface of the ovulation fossa	—	No follicle visible	On the side a moderately large follicle near ovulation fossa	On the side a moderately large follicle, no sign of anything in ovulation fossa
Corpus luteum	—	A very small yellowish orange patch, the side of rupture of last old corpus luteum	Trace of old corpus luteum	Old corpus luteum tissue brown, no sign of any corpus luteum	Old corpus luteum	Old corpus luteum		No corpus luteum visible	—	Relatively large corpus luteum with a large red point of ovulation clearly to be seen in fossa	No corpus luteum visible	A dark orange coloured corpus luteum with blood vessels on surface	No corpus luteum visible
Uterus:													
Colour of folds in mucosa	Folds in mucosa browner than in C_{11} or C_6		Folds pale yellowish pink colour (lymph in folds ?)		Mucosa yellowish, less pink than the others		Ovulation	Folds brownish yellow		Uterine mucosa pale pink-yellow		Pinkish yellow and pale	
Appearance	Mucosa of folds slightly more turgid, but not so pronounced as in C_{11}		Turgid as in C_6		Uterus folds flabby			Folds flaccid, moderate amount of milky fluid in lumen; a mass of sperm, many still motile, a few dead (ejaculation must have been through cervix*)		Folds in mucosa		Folds not as turgid as in C_{11}, and seemed to have less lymph	
Weight (in g.) uterus + cervix	490		263		196			380		182		133	
Cervix:													
Appearance	Pale; a little fluid mucus on the extension of the os and in upper vagina; finger will just go through cervix		Pale; a little thin mucus on extension of the os, but only moderately sticky inside cervix; cervix would only just let finger through		Congestion in cervix running up into the uterus; very thin mucus on surface; finger goes through quite easily			Congested, little thin mucus; finger can go easily through cervix; lot of sperm but not motile		Trace of moderately thick mucus, finger goes through easily. A tumour in side of cervix removed		Pale, a little moderately thick mucus on surface; aperture quite small and not possible to fit finger in	
Vagina and vestibulum vaginae:													
Weight (in g.)	319		200		234			263		235		—	
Appearance	Pale; a little fluid mucus on the outside of the os and in upper vagina		Pale		Pale			Very slight congestion in the upper part; in the middle part, thick mucus with a quantity of motile and dead sperm		Pale		Pale; a little moderately thick mucus in outer os	
Udder:													
Weight (in g.)	1040		512		507			564		245		499	
Appearance	Opaque, milky fluid, from teats. Yellowish colour		About 20 c.c. clear slightly yellowish fluid in nipples. Small globules (fat or leucocytes) and colostrum-like corpuscles, with trace of sticky mucus		No fluid in teats			No fluid in teats; mammary tissue well developed		Small, no fluid in teats		About 5 c.c. of clear, slightly yellowish fluid from nipples (of the same nature as fluid from C_{11})	

* Was killed immediately after service.

(Facing p. 16)

TABLE 5. HISTOLOGICAL APPEARANCE OF REPRODUCTIVE ORGANS OF MARES KILLED AT DIFFERENT PERIODS OF OESTROUS CYCLE

Stage of oestrous cycle ...	2 days before heat	1st day of heat	5th day of heat	6th day of heat	2 days after end of heat	8 days after end of heat
No. of mare	C_4	C_{11}	C_9	C_8	C_7	C_6
Extremitas ovarica of Fallopian tube:						
Epithelium	Cubical	As C_4, but larger in size, with leucocytes in stroma	Cells swollen; seemed to be filled with a secretion?	Same shape, may be filled partly with mucus (?); some leucocytes	More cubical in size	Cells cubical
Blood vessels	Relatively small	Normal, but subepithelial ones enlarged	As C_{11}	Seems to be congested	Small subepithelial; perhaps a little more enlarged than in C_4	As C_7
Horn of uterus:						
Epithelium	Cells cubical; surface of endometrium broadly folded	Cells as C_4; surface of endometrium folded in longer but narrower pleats	Cells swollen, longer shaped; endometrium surface fairly smooth	Cells columnar; surface smooth, practically without secondary folds	Cells columnar, higher than in C_4; folds marked again	Cells columnar, well marked folds
Glands	Not very numerous, many of them closed; interstitial connective tissue with some leucocytes	More numerous than before with a more open lumen; walls of glands thicker, with many infiltrated leucocytes	As C_{11}, but with more leucocytes in the subepithelial stroma	Numerous glands open, with thicker walls; lumen partly open. Blood vessels in stroma enlarged, partly extravasated	Glands not so large, mostly closed with a few leucocytes; small capillaries just visible	Considerable increase in number of glands which are smaller than before; practically without lumen. The increase in number of glands leads to a reduction of the stroma of connective tissue
Cervix:						
Epithelium	Cells mostly cubical, some of them elongated, but the majority short. A layer of mucus, mostly outside the cells. Fewer secondary folds, which are narrower than during heat and in the C_7 stage	As C_4, but epithelial cells have more lucid protoplasm; mucus layer more continuous	Cells swollen, rather columnar, partly filled with mucus; outside there is a wider mucus layer than in C_{11}	Shape of swollen cells as in C_9; much mucus deep in the bodies of the cells; a quantity of free mucus comes away from the surface of the lumen. Many folds; extravasation and congestion of blood vessels in stroma, probably due to foregoing coitus	Cells more cubical, but wider than before; longer than in C_4 with more mucous, mostly inside the cells, but with a small quantity outside the cells; numerous secondary folds, which are swollen and wider than e.g. in C_4. Muscles and connective stroma softer than in preceding stages	Cells notably shorter than before. Mucus mostly inside the cells; a very little outside
Height of epithelium	9·6	11·4	16·1	17·4	16·7	7·7
Vagina:						
Vagina proper:						
Epithelium	Normal and thin. Fewer secondary folds than in C_9	An increase in number of epithelial layers; external cells with the beginning of cornification	Large increase in thickness of cornifying epithelium. Secondary folds larger than before	Thickness of cornified epithelium increased, some cells flaking off. Leucocytes passing through epithelium	Large part of external cells lost, but still thicker than in C_4 and even C_{11}; fewer cornified cells. Leucocytes still passing through epithelium	Thinner layer than in the foregoing stage; fewer cornified cells than e.g. in C_{11}; a regeneration of epithelium that has been previously cornified and later shed
Blood vessels	Normal	Larger vessels congested; easily visible	Small capillaries congested. Some blood free under epithelium	Larger quantity of blood and more congestion (due to coitus before killing)	Less and less congestion and less blood than before, but still more than in C_4	Capillaries just visible
Vestibulum vaginae:						
Epithelium	Normal; low, with at most 1 or 2 layers of cornifying cells; some leucocytes pass through	More pronounced cornification; subepithelial tissue infiltrated with leucocytes	Thinner, due to more advanced cornification and flaking off of the epithelium; leucocytes still seen under the epithelium	Thicker than e.g. in C_4. Surface rough; cornified layers flaking off (more intensity than in C_9); the nuclei have less chromatin	Thicker than in C_8; layers of epithelium cells but fewer desquamating cells (beginning of regeneration?); surface fairly smooth	Thinner than before, fewer cornified layers of superficial cells
Blood vessels	Subepithelial capillaries just visible	Blood collected in capillaries; a little enlarged	Slightly more enlarged	Some congestion in subepithelial blood capillaries (due to the previous coitus?)	As in C_4	Subepithelial blood vessels slightly more visible than in C_7

mucus in the second part of oestrus must play the same important role of allowing the ascent of the spermatozoa through the cervix. This is confirmed by macroscopical observations (Day 1939*a*).

(*d*) *The vagina*

According to Schmaltz (1921*b*), Sisson (1930) and Williams (1931) the vagina in the mare represents the fused caudal ends of the Müllerian ducts and extends from the cervix to the vulva. The vagina is 30–32 cm. long and divided in two separate parts: the vagina proper (i.e. the part above the urethra) and the vestibulum vaginae (i.e. the part below the urethra). The hymen femininus marks the limit between these two parts of the vagina.

(1) *The vagina proper* forms, according to Schmaltz (1921*b*), three-fifths of the total length of the vagina and has a relatively simple structure: the walls are thin and consist of an adventitia, a muscularis and a mucosa. The muscularis, contrary to that of other domestic animals, is loose and consists of a middle part of smooth muscles, which is connected at both sides to connective tissue. The mucosa in turn has a layer of loose connective tissue and an epithelium 25–30 mm. high; the latter consists of four to five layers of polygonal cells with round nuclei.

Anatomical changes. Only very slight anatomical changes were observed during oestrus (table 4). In general appearance it is pale, except at one stage of the 6th day of heat (C_8). In this case the vagina was congested in its upper part, with thick mucus in the middle. This condition seems to be unusual, and is evidently due to a coitus previous to killing this animal. In the cow at the same period this condition is present—the vagina being congested and deeper in colour. The small amounts of mucus at different stages (C_4, C_8 and C_6) are probably produced by the cervix. But it seems likely that the portion of the vagina next the os undergoes very similar changes to those that occur in the cervix, as was stated in the case of the cow (Hammond 1927).

More obvious are the other changes in the vagina proper, e.g. the larger permeability of the blood vessels during oestrus and the increase in size and number of the secondary folds of the mucosa. The changes occurring in the vascularity of this area at different stages during the oestrous cycle are shown in table 5. The small subepithelial vessels, which enlarge at the beginning of the cycle, are, at the end of heat, congested and some blood can be seen free under the epithelium. After oestrus, on the 2nd and 8th days after the end of heat, the congestion of these small vessels is less pronounced, but on the 2nd day after the end of heat (C_7) they are still larger than 2 days before heat. In conclusion it may be noticed that the congestion of the blood vessels in the mare during oestrus occurs later and is less pronounced than in the cow.

Histological changes. The whole surface of the vagina proper is thrown up into a large number of primary and secondary folds. During oestrus, especially towards the end of heat, the number of secondary folds, as well as their size, increases.

More marked, as can easily be seen from table 5 and figures 14–19 (plate 3), are the changes in the epithelium: 2 days before heat (C_4) the epithelium is thin, with only one dark stained and one cornified layer. On the 1st day of heat the secondary mucosa folds are more marked, and there is a beginning of cornification (C_{11}). On the 5th and 6th days of heat these changes are developed even further (C_9 and C_8), with very distinctly marked folds of the mucosa and a thick, well-cornified epithelium. On the last day of heat the layers of epithelial cells are thrown off and a number of leucocytes wander through the epithelium.

At the end of heat the epithelium is thinner, but, on the 2nd day after the end of heat, it still remains thicker than it was before heat. The shedding of cornified cells continues, although not in such great quantity as at the end of heat; a few leucocytes still find their way through the epithelium. In the last stage, i.e. 8 days after the end of heat, there is a regeneration of the epithelium, although it has not yet assumed its pre-oestrous thickness.

In conclusion it may be pointed out that the anatomical changes in the vagina proper during the oestrous cycle are in the mare less pronounced (especially in the lack of bleeding) than in the cow. Nevertheless, the histological changes (i.e greater cornification) are more pronounced in the mare than in the cow. The shedding of the vaginal epithelium and leucocytosis in the mare are processes similar to those observed during the oestrous cycle in some rodents.

(2) *The vestibulum vaginae.* The vagina below the urethra constitutes the posterior termination of the genital canal. This portion forms, on the average, two-fifths of the total length of the vagina. According to Schmaltz (1921*b*) the erectile tissue in the submucosa is less pronounced in the mare than in other domestic animals. The flat stratified epithelium originates from the ectoderm and forms many layers, so that it is twice as thick as in the vagina proper. The most particular feature is the growing of the epithelium down into the mucosa, like the lacunae Morgagni in the human urethra; it may or may not have a visible lumen.

The anatomical changes were described in connexion with the vagina proper.

The histological changes observed in different stages of the oestrous cycle are reported on in table 5 and figures 18–21. The long storage of our material in formalin solution was no help in the cutting and staining of the preparations.

The vascular changes are similar, although it seems to be less affected by a congestion of the blood vessels during the cycle. There is an enlargement of the subepithelial capillaries at the beginning of the heat period (C_{11}). Afterwards their size further increases; they are always numerous. Probably they are connected with the vascular supply to the erectile organs.

More significant are the changes in the epithelium. As is shown in table 5 and figures 20–23 (plate 4) the chief characteristics are changes in thickness and in amount of cornification. Furthermore, it must be pointed out that the surface of the lower vagina mucosa consists of two different parts: in some places it is like the rugae vaginales in the human subject, in other places it has a smoother surface without folds (figures 23 A, B and C). The thickness of the epithelium is also affected by the presence of the above-mentioned lacunae which sometimes form 'pearls', i.e. peculiar inclusions in the deeper layers of the stratified epithelium.

At the beginning of oestrus (C_{11}) there is an increase of cornification and a larger accumulation of leucocytes in the connective tissue beneath the epithelium.

On the 5th day of heat (C_9) the cornification is still more advanced; epithelial cells are thrown off and leucocytes start penetrating the epithelium.

On the 6th day of heat (C_8) the shedding of cornified epithelium cells is even more pronounced than on the 5th day: the surface of the epithelium is much thinner and becomes rough by the shedding of several cornified layers.

After the end of heat (C_7 and C_6) the epithelium regenerates and grows up. Very few cells are shed and, at the end of this period (i.e. 8 days after the end of heat), it has a normal appearance with a few cornified cells.

In conclusion it can be stated that the most important changes in the vestibulum vaginae during the oestrous cycle are represented by a relatively feeble increase in the size of the blood vessels, and a well-marked cornification of the epithelium and an increase in the depth of the stratified epithelium. This is in contrast to the cyclic changes of the cow, where the congestion of the blood vessels, with even extravasation of blood beneath the surface of the epithelium, are the most prominent features (Hammond 1927).

(*e*) *The mammary gland*

The structure of the udder in the mare is described by Schmaltz (1921 *b*) and Williams (1931).

Owing to circumstances beyond our control, we can only give a short statement of the anatomical changes observed during the oestrous cycle (table 4). We have not been able to come to any satisfactory conclusions, as there is a considerable range of variations in individuals, and we had only a few experimental mares. In one of our mares (C_8) killed some time after parturition, there was some increase in the weight of the udder. The figures in table 4 should, however, be helpful to further investigations with more material.

SUMMARY AND DISCUSSION

A study of the changes in the reproductive organs of the mare during the oestrous cycle has been made by the observations of changes in the weight, anatomy and histology. Although the material was barely sufficient, its great uniformity as mentioned above allows us to draw the following conclusions.

The length of the cycle and the duration of oestrus shown by the experimental mares (which had been regularly examined for a long period previous to killing) corroborates the results of Hammond (1934, 1938*a*,*b*) and Day (1939*a*,*b*, 1940). In our mares the length of heat was 7 days, and the length of dioestrus 16 days. The degree of constancy in the length of the oestrous period in our case is probably due to the uniformity of our material, as well to time of year at which all the animals were killed.

Contrary to a statement of Kupfer (1928) (concerning the donkey) ovulation takes place in the ovulation grove. The ovulation occurs at some time during the last days of heat; thus the previous findings of Hammond (1938*b*) and Day (1939*a*) are confirmed.

The principal phenomena occurring in the ovaries of the mare during the oestrous cycle are shown by measurements of the size of the active and inactive ovaries, the follicles and corpora lutea. It can be stated that, in the mare in contrast to the cow (Hammond 1927) and the sow (Corner 1921), the size of the ovary during the oestrous cycle is chiefly influenced by the growing Graafian follicle.

A survey of the development of the Graafian follicles and corpora lutea was undertaken. The average diameters of the follicles and corpora lutea have been recorded and plotted in curves. In contrast to the cow and the sow, where there is a gradual growth of the follicle, we have in the mare a very sudden one. Furthermore, the number of follicles, which are present at different stages, varies greatly. Each of the curves showing the growth of the immature, small follicles, follows to a certain extent that showing the development of the large Graafian follicle; in this the mare differs from the sow (Corner 1921). It is suggested that the abundant small follicles at the beginning of heat, which disappear later and probably partly degenerate, do so on account of an absence of follicle-stimulating hormones from the anterior pituitary. Liche (1939) has stated that, in the cat, the follicles undergo a process of athresia.

The formation of a new corpus luteum, as well as its subsequent decrease in size during later stages of the cycle, is described. The colour of the newly formed corpus luteum varies greatly during its life from a pale tint to brownish, or orange and scarlet. As in the cow the rupture of the follicle is associated with some bleeding on and under the surface of the ovulation fossa; later this is shown as a chocolate-coloured spotting. The point of rupture can be seen during the following days as a large point of ovulation in the ovulation grove. It can be stated that the active stage of the corpus luteum is very short, much shorter than in the sow (Corner 1921) and cow (Hammond 1927). These latter have also a comparatively long period of maximum corpus luteum development, whereas in the mare the active stage is very short, and the maximum diameter of the corpus luteum seems to be always below that of the Graafian follicle.

The larger increase of the Graafian follicle with its secretion of oestrin in the mare plays a more important role than in the cow or sow. As the spontaneous ovulation in the mare occurs exclusively in the ovulation fossa, the heat is much longer: this would appear to be due to the longer time taken by the follicle to come to the surface of the ovulation fossa and break through.

The changes occurring in the Fallopian tubes, uterus, cervix, vagina proper and vestibulum vaginae are described.

The changes occurring in the Fallopian tubes during the heat period are less pronounced than stated by Courrier (1930) in dogs, bats, swine and guinea-pigs. There is a slight enlargement both of the subepithelial blood vessels and of the epithelial cells. The secretion stage of the latter is not pronounced.

Some anatomical and histological changes occur in the uterus during the cycle. With reference to the former there are changes in colour as well as in the turgidity of the uterine folds. The histological changes concern the permeability of the walls of the blood vessels, the shape of the epithelium cells and glands, and the surface of the mucosa. It is considered that the cyclic changes in the uterine mucosa of the mare differ from such species as the rabbit, ferret and cat, where there is a very specialized placentation, in that they are far less marked. They are also less marked than in the cow; rather are they similar to species with a simple placenta, such as the sow (Corner 1921).

The presence of semen in the uterus of one of our mares, killed immediately after service, shows that the stallion in this case ejaculated into the uterus. This throws some light on what is, from the breeding point of view, important (Day 1939*a*), that is, that the cervix must be relaxed before this can occur.

Marked changes occur in the cervix during the cycle. Anatomical changes concern the degree of congestion of the blood vessels and the amount of opening of the lumen, but both anatomical and histological changes are less pronounced than in the cow (Hammond 1927). The cervix acts as an important mucous gland during the whole cycle, allowing the ascent of the spermatozoa, but only towards the end of the heat period is there a distinct flow.

The structure of the vagina proper and the vestibulum vaginae is described; in the mare it varies in different parts of the vagina. The anatomical changes that occur during the oestrous cycle are far less pronounced than in the cow, as there is no bleeding. Histological changes in both parts of the vagina are represented by a well-marked cornification of the epithelium and an increase in depth, especially towards the end of heat. During metoestrus, after the cornified cells are shed, there is a regeneration of the epithelium. There is no such cornification in the cow, but there are similar processes in some rodents, where there is also a marked leucocytosis.

Certain anatomical changes in the mammary gland during the oestrous cycle are described but not discussed.

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Photosynthesis in intermittent illumination

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The reduction of one molecule of carbon dioxide per two thousand molecules of chlorophyll after each intense flash of light of very short duration, while three hundred or more are reduced when the duration of the flash is greatly increased, can be explained by assuming that chlorophyll sensitizes the activation of a substance A which, by handing on its energy, causes the reduction of carbon dioxide in combination with a substance S. The decay of activated A is a relatively rapid process while that of the activated compound of carbon dioxide and S is relatively slow. The yield for short flashes is determined by A, that for long flashes by S. The ratio of S to A is of the order of 100.

The experiments of Emerson and Arnold (1933) showed that a suspension of *Chlorella* cells in a medium rich in carbon dioxide, illuminated by short flashes of light of about 10^{-5} sec. duration separated by dark periods of 0·1 sec., reduced one molecule of carbon dioxide per flash for every 2000 or so molecules of chlorophyll present. This yield per flash is the maximum and is approached as the intensity of the flash is increased. Kohn (1936) has reported that the intensity of the flash to give half-maximal yield is $7 \cdot 2 \times 10^{12}$ quanta per cm.^{2}, and that the yield, Y, is related to the intensity of the flash, E, by the equation

$$Y = Y_{\text{max.}}(1 - e^{-KE}),$$

K being $9 \cdot 7 \times 10^{-14}$ cm.^{2}/quantum. This quantity is 360 times the molecular extinction coefficient of chlorophyll for the light used. These results have led to the suggestion that there are four to six hundred molecules of chlorophyll for each centre capable of combining with carbon dioxide and that four quanta of energy are required for the reduction of each molecule of CO_2.

It has been pointed out (Briggs 1935) that it is difficult to reconcile the results of Warburg (1919) with such a theory. In these experiments with a suspension of *Chlorella* the intermittent illumination consisted of periods of light of the same length as the periods of darkness. With high intensity the rate of photosynthesis for intermittent illumination was above half the rate with continuous illumination and became practically the same as the speed of intermission was increased. Warburg interpreted his results as indicating that the rate of photosynthesis was greater in the light period of intermittent illumination than it was during continuous illumination, the greater rate being attributable to a greater concentration of a complex of carbon dioxide with a substance in the plant cell. This may partly explain the results of experiments where the concentration of carbon dioxide in the supply is small, as is the case in the investigation of McAlister (1937), but Warburg's experiments were carried out with a concentration which was probably so high that further increase would have had little effect on the rate. Hence Warburg's explanation does not seem to be likely. None of the recent attempts to formulate mechanisms for photosynthesis appear to have taken these results into account. Wohl (1937), for example, merely considers the limiting case where the intermission is very rapid.

We have repeated experiments of the Warburg type with variations, under conditions where the supply of carbon dioxide definitely did not limit the rate of photosynthesis.* The variations were planned to bridge the gap between experiments of the type of Warburg and that of Emerson and Arnold. Our results are in agreement with those of Warburg, and in view of the conditions of carbon dioxide supply it seems more reasonable to interpret the excess of the rate in intermittent light over half the rate in continuous as being due to a continuation of photosynthesis during the dark period. The term yield will be used for this excess of assimilation over that which would be obtained if the process continued during the light periods at the same rate as in continuous illumination but stopped completely during the dark intervals.

Whereas the yield per flash obtained by Emerson and Arnold represents 1–2 % of the amount of carbon dioxide reduced in one second of continuous illumination of high intensity, the yield in experiments of the Warburg type attains a value of 500 % or more, and the length of the dark period for such a yield is more than 10 sec. It was suggested earlier (Briggs 1935) that flashes of high intensity of longer duration than 10^{-5} sec., but still short compared with the dark period, should give useful information. Emerson and Arnold stated that lengthening the flash to about 10^{-3} sec. without changing the energy content resulted in little change in the yield, but these flashes were probably not of sufficient intensity to give the maximal yield. In any case they did not try the effect of increasing the intensity of these flashes of longer duration. Our experiments with a single sector wheel giving flashes of short duration with a dark period about ninety times as long are not easy to interpret. To obtain a high intensity of illumination a convergent beam from a large mirror was used with the result that although the bright part of the flash lasted only about 4×10^{-4} sec. the total duration was a good deal longer, the dark period being about 3×10^{-2} sec. Under these conditions the rate of photosynthesis was the same as that with continuous illumination of high intensity, thus indicating a yield of about 3 %. With an arrangement of two sector wheels more clearly cut flashes were obtained, but the shortest that could be secured was 0·0342 sec. With a dark time of 0·602 sec. the yield for the most intense illumination (1–2 million lux) was much greater than that recorded by Emerson and Arnold and reached 8–10 %. When the duration of the flash was lengthened without changing the intensity the yield increased, until with a flash as long as the dark period the yield rose to nearly 60 %. In other words, the rate in intermittent light was practically the same as in continuous. Greater yields were obtained by lengthening still further the light and dark periods. It is clear that these results are not consistent with a theory such as that referred to at the beginning of this paper.

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* These experiments have been carried out during the past five years by L. A. T. Ballard and B. G. Wilkinson in the Sub-Department of Plant Physiology at Cambridge. Both of these research workers are now on National Service and it will be impossible for some time to give full details of their experimental work.

The following schema seems to account for these results and those recorded by Emerson and Arnold. We assume that the energy absorbed by chlorophyll is passed on, not directly to S_c, the complex of carbon dioxide with a cell substance S, but *via* a substance A. The activated form of A, A', reacts with S_c to give S_c' and normal A, and S_c' gives rise directly, or *via* a catalyst, to the products of photosynthesis and free S substance, which can then recombine with carbon dioxide. If it were not that chlorophyll is assumed to be activated first, our A' would be the photochemical primary product of Warburg (1920). At present we are not concerned with the chemical nature of A', whether A is merely increased in energy content or reduced to a form AH (see later). A similar schema was suggested recently by Ornstein *et alia* (1938), but whereas they assume that the substance A 'is in abundance' our suggestion is that the ratio of A to chlorophyll is of the order of 1/500. Their suggestion that A is in abundance is to account for the high efficiency of the photosynthetic process at low intensities of illumination. As Wohl (1940) has indicated, there are other ways of accounting for this high efficiency.

Dealing with conditions of high concentration of carbon dioxide and putting A for the concentration of total A, normal and activated, and similarly S for the concentration of S_c and S'_c together, we have

$$dA'/dt = k_1(A - A') - k_2A'(S - S'_c),$$

$$dS'_c/dt = k_2A'(S - S'_c)/4 - k_3S'_c.$$

The constant k_1 will depend upon the intensity of illumination and upon the number of chlorophyll molecules which can hand on energy to each molecule of A. The variation in the efficiency of photosynthesis at low intensities of illumination suggests that in some cases part of the chlorophyll is not in a position to hand on the energy it has absorbed. The constant k_3 may, as suggested elsewhere (Briggs 1935), be a function of the concentration of a catalyst and of the concentration of S'_c. We are not now concerned with the question as to whether A undergoes four stages of activation before activating S_c or the latter substance is activated in four separate steps. In the above formulation we have taken the latter alternative and have assumed that all the three lower stages of activation of S_c are equally effective as acceptors.

For very brief flashes even of high intensity the results indicate that the fraction of S_c activated per flash is not great; that is, S is great compared with A. Hence during brief flashes separated by long dark periods, but not long enough for S'_c to change appreciably*, we have

$$dA'/dt = k_1(A - A') - k_2A'pS,$$

$$A' = \frac{k_1A}{k_1 + k_2pS}[1 - e^{-(k_1+k_2pS)t}].$$

The fact that the rate of photosynthesis in flashing light of this kind is about one-sixth of that in continuous illumination suggests that p in the above equations is about 5/6. The total energy handed on to S_c during a flash and ensuing dark period long enough for the transfer to be almost complete will be

$$\frac{k_1Ak_2pS}{k_1 + k_2pS}T + \frac{k_1^2A}{(k_1 + k_2pS)^2}[1 - e^{-(k_1+k_2pS)T}],$$

where T is the duration of the flash. This equation becomes the same as that by Kohn (p. 24) when $k_1 \gg k_2pS$ and T is small. With increase in the intensity of flash the maximum transfer of $k_2pSAT + A$ will be approached.

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* As shown later the velocity constant for the breakdown of S'_c is relatively small.

The experiments of Emerson and Arnold suggest that k_2pS is of the order of 100, so for a flash of 10^{-5} sec. the maximum yield* is equal to A. Therefore, one molecule of carbon dioxide for each four molecules of A is reduced per flash, since the former requires four quanta. If the duration of the flash were increased to 10^{-3} sec. without altering the energy content of the flash the yield would be increased by 10 % provided k_1, now reduced to 1/100 of its previous value, is still very great compared with k_2pS. According to Kohn's results (1936) with an intensity of flash (duration 10^{-4} sec.) giving half the maximal yield the rate of absorption of quanta is about 20 per sec. per chlorophyll molecule, which is about 10^4 per molecule of A. A flash of such intensity spread over 10^{-3} sec. would give k_1 a value of 1000 and a yield practically the same as with the shorter flash. As stated earlier, we do not know the intensity of the flashes which Emerson and Arnold used when they tested the effect of prolonging the flash to 10^{-3} sec., but the context suggests that when of short duration the flashes were not intense enough to give maximal yield.

Turning to the results of our experiments, the intense flashes of duration 0·0342 sec. gave a total yield, including the assimilation during the flash, ranging from 10·5 to 15·2 %. Taking k_2pS as 100, as before, the total yield should be equal to 4·42 $A/4$. Actually the average value of p would be somewhat smaller than in the previous case, since the average rate of photosynthesis in the intermittent illumination was about one-quarter of the rate in continuous. A reduction of the duration of the flash without reducing the intensity should, in the limit, reduce the yield to a value ranging from 2·4 to 3·5 %, that is $A/4$. This value is not very different from that obtained by Emerson and Arnold with brief intense flashes (0·8–2·2 %). Moreover, it should be remembered that expressed as a percentage, as above, the yield is not fixed, but depends upon A/k_3S. So it can be concluded these results are not inconsistent with those of Emerson and Arnold if interpreted on the basis of our schema.

As the duration of the flash is lengthened until the concentrations of A' and S'_c approach those of the steady state attained in continuous illumination, the yield in the dark will approach the value of the steady state value of S'_c if the dark period is long enough for the whole of S'_c to breakdown. For high intensities of illumination and high concentration of carbon dioxide

$$A' = A \quad \text{and} \quad S'_c = \frac{k_2 AS/4}{k_2 A/4 + k_3}.$$

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* With flashes of very short duration the yield is practically the same as the transfer of energy.

If the dark period is not long enough, the yield will be only a fraction of S'_c; a fraction depending on the duration of the dark period and the order of breakdown process. The point to be noticed here is that the maximum yield for long flashes with long dark periods is determined by S, the substance which combines with carbon dioxide, while that for very brief flashes is determined by A. The variation of yield with length of dark period will be discussed later along with the full experimental results.

As stated earlier, with a dark period of 0·602 sec. the yield increases as the duration of the flash is increased until a value of about 60 % is attained when the duration equals that of the dark interval. When both periods are further increased, the yield attains yet higher values. It requires a time of the order of 10 sec. for half the maximal yield to be secured, as compared with 1/100 sec., the time for half maximal yield when the light flashes are of 10^{-5} sec. in duration. This indicates a value for k_3 about 1/15 and a maximal yield with long flashes equal to 1500 % if the breakdown of S'_c is unimolecular; the rate in continuous illumination is $k_3S'_c$, and the yield S'_c. Since the estimation of the yield depends upon the difference between the rate in continuous illumination and twice the rate in intermittent illumination (the rate per second of actual illumination), the estimate becomes less certain as the yield becomes greater and the intermittent rate approaches a value equal to half the rate in continuous illumination. For the purpose of estimating the ratio of S to A we shall take a value of 1000 % as the yield for long flashes and 3 % for very brief flashes. In terms of our schema the ratio of the two yields is $k_2S/(k_2A/4+k_3)$. Taking k_2S as 100 and k_3 as 1/15, we get $k_2A = 14/15$ and the maximum yield for long flashes $7S/9$. The ratio of S to A is about 100 and remains the same if we assume that A receives all the four quanta before it can activate S_c. We made no determinations of the amount of chlorophyll in our material, but in as far as it is comparable with that used by Emerson and Arnold the ratio of S to chlorophyll is about one to five. It may be noted in passing that if the temperature coefficient of k_3 is greater than that of k_2, this schema provides an explanation for the variation of the temperature coefficient of the rate of photosynthesis with material as due to a variation in the A/S ratio and also for the fall in the coefficient as the temperature is increased and the ratio k_3/k_2 is increased.

In all these experiments and those on *Chlorella* the reduction of carbon dioxide was measured in terms of the oxygen produced. McAlister (1939) has reported a 'pick-up' of carbon dioxide by wheat leaves on the termination of illumination. This uptake with an atmosphere containing 0·3 % carbon dioxide amounts to one molecule to 2–4 molecules of chlorophyll present, and only a small part of this uptake can be attributed to carbon dioxide going into solution in the leaves. Expressed in terms of the amount of assimilation in a second the uptake is about 1000 %, a value comparable with our maximum yield. Moreover, the time curves for carbon dioxide pick-up are similar as regards time scale with ours for output of oxygen. This likeness is to be expected if the breakdown of S'_c, giving oxygen, released free S substance which then combined with carbon dioxide as we have suggested. If pick-up is a measure of the amount of S'_c at the end of the period of illumination, then the curve given by McAlister for the relation between rate of photosynthesis at different intensities of illumination and subsequent pick-up is of the non-linear type to be expected if the breakdown of S'_c is not a unimolecular process but one determined by combination of S'_c with a catalyst. Other evidence for this type of breakdown has been discussed earlier (Briggs 1935).

The statement that, after a period of illumination in air free from carbon dioxide, the introduction of carbon dioxide on darkening did not result in a pick-up of carbon dioxide, suggests that only when the S substance is freshly liberated is it capable of combining with carbon dioxide. If we assume that this active form, S_a, can combine reversibly with carbon dioxide to form S_c, the velocity constants of formation and dissociation being relatively great, or alternatively change reversibly to an inert form S_b, the velocity constants being relatively small, but the ratio of the constants which decides the ratio of S_b/S_a at equilibrium relatively great, then, not only should we have a mechanism in harmony with the above facts, but also one in agreement with the observation that after a period of darkness, during which S will proceed mainly to the S_b form, return to illumination results in a phase of increasing rate of photosynthesis as more and more of S_b is converted *via* S_c to S'_c. The velocity constant for the conversion of S_b to S_a being small, the rise in rate of absorption of carbon dioxide would be determined chiefly by this process whatever the intensity of illumination, but the induction loss (the difference between the absorption if the rate had been maximal from the outset of illumination and the actual amount) would be closely related to the final value of S'_c, that is, to the rate of photosynthesis. This is in agreement with the results of McAlister. The statement by McAlister which is not in agreement with the above picture is that after a period of illumination in air free from carbon dioxide the rate of photosynthesis commences at a normal rate on the introduction of carbon dioxide. McAlister, who makes no reference to the possibility of the carbon dioxide pick-up being connected with oxygen output, goes as far as stating 'the material reponsible for the pick-up is an intermediate formed only during photosynthesis'. The suggestion is not given a more concrete form and the difficulty of reconciling the last observation with the others is not considered.

Recently Hill (1939) and Hill and Scarisbrick (1940) have investigated the production of oxygen by the reduction of ferric to ferrous iron in the form of oxalate by illuminated chloroplasts of *Stellaria* isolated from the leaf cells. The relation between rate of production of oxygen on the one hand and intensity of illumination, temperature, and concentration of depressants such as potassium cyanide and urethanes on the other shows a close parallelism with the relation for photosynthetic reduction of carbon dioxide by the intact leaves. Hill's suggestion is that a substance A is reduced to AH_2 with the evolution of oxygen and is then oxidized back to A by the ferric salt. A mechanism such as this with our S_c as the hydrogen acceptor could explain the results of our experiments on photosynthesis in intermittent illumination. In the case of flashes of long duration the extra assimilation per flash would be, in terms of carbon dioxide, an uptake in the dark period, but in terms of oxygen produced it would be as a higher rate of production in the earlier part of the light period. Since the photosynthesis is measured as the average rate of carbon dioxide consumption or oxygen production over a period including many flashes, the results would not distinguish between a mechanism such as Hill's and one such as ours where the oxygen is produced by a substance which then rapidly combines with the carbon dioxide. The evidence against the former type of mechanism for the photo-reduction of carbon dioxide is that when photosynthesis is measured as oxygen production there is no indication of a higher rate at the outset of illumination. Admittedly there may be complicating factors of the type we have already discussed which would obscure such a fall in rate of oxygen production.

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Competition between related strains of nodule bacteria and its influence on infection of the legume host

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The work deals with the behaviour of mixed strains of nodule bacteria towards each other and towards their legume host.

It introduces the concept of dominance in competition between strains. This dominance is independent of degree of effectiveness as regards nitrogen fixation.

Where two strains of nodule bacteria are both present in the surroundings of their host's root system, active competition between them may cause the strain having the higher initial growth rate almost completely to check multiplication of the other strain outside the plant. This dominant strain will then be responsible for nearly all the nodules.

In peas and soy beans, where growth of the root system is rapid and of comparatively short duration, the nodule-producing capacity of the plant may be partially or wholly satisfied by the nodules produced within the first few weeks, so that further infection, whether by the same or by a different strain, is checked or inhibited.

In clover, whose root system continues to grow over a long period, the first-formed nodules do not stop further nodules from being formed either by the same or by a different strain.

There are large differences in the rates of appearance and final numbers of nodules produced by different strains supplied in pure culture, particularly with clover.

The relative numbers of nodules produced by the two strains simultaneously applied to the roots is conditioned by the specific infectivity peculiar to each strain, unless some other factor, such as competition outside the plant, masks this effect.

1. INTRODUCTION

The factors that control the infection of crop plants by various micro-organisms have been extensively investigated in the hope of being able to check infection by pathogens and of stimulating it in the case of symbiotic organisms. Most of these studies have been carried out with pure cultures, although in a state of nature the micro-organism attacks its host plant in the presence of, and often in competition with, other organisms which may belong to quite different groups or to strains closely related to itself. The

Competition between different genera and species of pathogenic and saprophytic fungi in the soil has formed the subject of fruitful study. For references, see a review by Garrett (1939).

Very little is known, however, about the intra-specific competition for infection that may take place when two or more related strains of a pathogenic or symbiotic organism are present, either simultaneously or in succession, in the surroundings of their host plant. This type of competition, when it takes place between strains differing markedly in their effect on the host, must greatly influence the results of infection, and presents a problem of evident importance both to plant pathology and to the practical application of our knowledge of the symbiotic nodule bacteria.

It is a complex problem involving factors such as competition *outside* the host plant, as well as the relative infective virulence of the strain and the question of a possible acquired immunity developed by the host plant: factors which may operate independently or may interact.

The genus *Rhizobium* offers unique material for the investigation of competitive infection because infection is localized in the form of nodules, a count of which provides a simple measure of infectivity; because it is often relatively easy to identify the strain that has produced a given nodule, and because the host is not killed by the infection which can hence be studied over the full period of the plant's growth.

A field of research of considerable agricultural importance has been opened up by the discovery that certain strains of *Rhizobium* produce nodules that are almost wholly ineffective in benefiting their legume host plant. In particular, this discovery suggests a new application for the process of legume seed 'inoculation' with cultures of nodule bacteria. Hitherto this process has been mainly successful where a leguminous crop, often of comparatively recent introduction, was being grown in a soil otherwise lacking or deficient in bacteria capable of producing nodules upon its roots. However, soils are now known where a legume crop finds a predominant strain of *Rhizobium* which will produce nodules upon it but will not materially benefit its growth. In such soils it should be possible to use the inoculation process to supply the legume crop with an effective strain to replace the ineffective one that it will otherwise acquire from the soil.

But if this replacement is to be a success, the introduced beneficial strain must be capable of competing for nodule formation with the ineffective strain already present, probably in far greater numbers, in the soil. Our information about strain 'effectiveness' will be of little use in extending the benefits of the inoculation technique to soil infested with ineffective strains until we have some understanding of the factors that control competition for nodule formation when two or more strains, both capable of forming nodules, are present in the root surroundings of the host plant.

The important effects of this competition upon the yield and nitrogen content of the host crop plant are well shown by some work carried out at Rothamsted with the ineffective 'Coryn' strain which infects clover. This strain was isolated from stunted clover plants which characterized the pastures on Coryn Mountain in central Wales. Preliminary experiments with clover grown in pots of sand indicated that when this strain was present in the sand it reduced the growth of the plant even when effective strains were also present (see experiment 1). A search was therefore made for an effective strain capable of producing a good growth of clover in sand containing Coryn strain bacteria. A strain satisfactory in this respect was obtained from Professor Ch. Barthel, Central Agricultural Experiment Station, Stockholm, for whose kind assistance thanks are due. This strain is referred to below as strain A.

The following experiment illustrates the effect of the Coryn strain on the growth of clover in the presence of strain A and of a mixture of strains of clover *Rhizobium*, effective by themselves but liable to be suppressed when in competition with the Coryn strain.

Experiment 1

Montgomery red clover was grown in glazed earthenware pots each containing 12 kg. of nitrogen-deficient sand. One litre of the following nutrient solution was added:

K_2SO_4	0·9 g.	$MgSO_4.7H_2O$	0·5 g.
$CaSO_4.2H_2O$	0·3 g.	NaCl	0·5 g.
K_2HPO_4	0·3 g.	$FeCl_3$	0·04 g.
KH_2PO_4	0·3 g.	Tap water	1 l.

All the pots received a mixed inoculum of efficient strains of clover nodule bacteria derived from Rothamsted soil.

The sand was not sterilized because it was hoped that wild strains of bacteria casually introduced might contain amongst them some that were capable of competing effectively with the Coryn strain. This hope was not realized, but later, a strain having this desirable character was isolated from Agdell Field, Rothamsted. This strain (labelled W) was included in experiment 2.

Set 1 received only the wild local strains, set 2 received in addition a suspension of the Coryn strain, set 3 of strain A, and set 4 of both these strains in approximately equal numbers. These inocula were added to the food solution before it was added to the sand. Set 1 consisted of five and the remaining sets of fifteen replicate pots; each pot contained five plants. The seed was sown on 11 April. The resulting dry weights and nitrogen contents of the plants obtained after 14 weeks' growth, are shown in table 1.

TABLE 1. EXPERIMENT 1. RED CLOVER GROWN IN NITROGEN-DEFICIENT SAND

set	no. of pots	strains of *Rhizobium* supplied	mean dry weights per pot g.	standard errors	N content of plants, means per pot mg.	standard errors
1	5	mixed Rothamsted strains only	28·00	± 1·32	701	± 34·4
2	15	mixed Rothamsted strains plus Coryn strain	8·95	± 1·91	218	± 46·3
3	15	mixed Rothamsted strains plus strain A	26·84	± 0·85	673	± 32·7
4	15	mixed Rothamsted strains plus Coryn strain and strain A	21·89	± 1·57	550	± 40·7

The mixed local strains without further addition (set 1) produced a good growth of clover with a mean dry weight per pot of 28 g. containing 701 mg. of nitrogen. The addition of the Coryn strain to these mixed strains in set 2 reduced their effect so that the clover in set 2 gave a mean yield of 8·95 g. containing 218 mg. of nitrogen. But in the presence of strain A, the Coryn strain reduced the growth and nitrogen fixation to a very small though significant extent (set 4).

Experiment 2

The effects on the growth of red clover of the effective strains A, W and the Wisconsin strain 205 alone and in combination with the ineffective strains Coryn and Wisconsin strain 202 were tested in a pot experiment in nitrogen-deficient sand. The two Wisconsin strains used were amongst those employed by Dunham and Baldwin in their work on double inoculation (1931). The clover was grown in glazed earthenware pots containing 12 kg. of sand supplied with the same nutrient solution as was used in experiment 1.

Sets of pots were supplied with each of the effective strains alone and in combination with each of the ineffective strains. Suspensions of the bacteria were added to the nutrient solution before pouring on to the sand. In the sets receiving two strains, approximately equal numbers of cells of each strain were mixed in suspension before addition to the sand. The sand was not sterilized because the previous experiment had shown that it did not contain strains capable of competing with the Coryn strain. Montgomery red clover was sown on 23 March and grown for 14 weeks. The dry weights and nitrogen contents then found are shown in table 2.

TABLE 2. EXPERIMENT 2. RED CLOVER GROWN IN NITROGEN-DEFICIENT SAND

set	no. of pots	strains of *Rhizobium* supplied	mean dry weights per pot g.	standard errors	N content of plants, means per pot mg.	standard errors
1	10	A	9·13	± 0·91	230	± 27·3
2	10	W	8·90	± 0·40	228	± 13·7
3	10	205	7·23	± 0·46	214	± 10·7
4	10	A + C	9·54	± 0·60	257	± 18·7
5	10	W + C	9·87	± 0·97	260	± 21·9
6	15	205 + C	0·29	± 0·23	4	± 1·0
7	10	A + 202	11·31	± 0·52	280	± 19·9
8	10	W + 202	9·85	± 0·75	244	± 20·5
9	10	205 + 202	5·08	± 0·62	162	± 13·9

The three effective strains produced a good growth of clover in the absence of the ineffective strains (sets 1–3). The normally effective strain 205 was almost incapable of supporting growth of the clover in the presence of the Coryn strain (set 6). The effect of strain 205 was also significantly reduced by the presence of the ineffective strain 202 (set 9). But the effects of the dominant and effective strains A and W were not significantly affected by the presence of the large inocula of Coryn or 202 added to the sand in sets 4, 5, 7, and 8. The results of these two experiments are most easily explained on the assumption that certain strains prevent or check nodule formation by other strains that are simultaneously present in the root surroundings. This check might be due either to competition between the strains outside the plant or to nodules first produced by one strain conferring an immunity in the plant against the entry of the other strain. Israilsky (1929) claimed that such an immunity could be induced.

Marie Löhnis (1930) made experiments with red clover inoculated with Wisconsin strains 205 and 202. She found that when the plants were supplied with one of these strains at sowing time, re-inoculation with the same or with the other strain after about a month's growth caused no significant increase in nodule numbers. She grew her plants in tubes of agar medium, under which conditions poor growth of the plant usually limits nodule numbers to a low figure which might well have been reached in month-old plants.

Dunham and Baldwin (1931) made experiments with lucerne, clover, peas and soy beans, using, with each host plant, an effective and an ineffective strain of nodule organism, the two strains being applied to the same plants, in some sets simultaneously at sowing time, and in other sets in succession, one strain at sowing time and the other after an interval of 4–6 weeks. After simultaneous inoculation, the two strains both usually produced nodules. With successive inoculation, in six sets out of eight the first-applied strain produced all the nodules that were tested, the remaining two sets giving nodules by both strains. In a second experiment using clover inoculated at sowing with strain 202 and after 56 days re-inoculated with strain 205, the first strain contributed all of the 100 nodules tested.

This preponderating effect of the first-applied strain could, on the evidence, be explained by several hypotheses:

(*a*) The growth of the plant may have been slowed down to such an extent by the time the second inoculation was made that nodule formation by either strain had ceased.

(*b*) The first strain may have continued to produce nodules after the second inoculation but it may have immunized the plant specifically against the second strain. This explanation is the one favoured by the authors.

(*c*) The nodule-bearing capacity of the plant may have been satisfied by the first-applied strain so that, by the time of the second inoculation, few or no further nodules could be produced by either strain. Neither this nor explanation (*a*) is sufficient to explain the results of Dunham and Baldwin's second experiment with clover, where they refer to the formation of nodules on new roots. But either explanation might apply to their results with other plants.

(*d*) The first strain may have swamped the second strain and prevented its multiplication *outside* the plant. In this work the second strain was poured on to the sand which already contained a presumably large population of the first strain.

2. FACTORS AFFECTING THE COMPETITION FOR NODULE PRODUCTION BETWEEN STRAINS OF PEA AND SOY BEAN *RHIZOBIUM*

Experiment 3

The following experiment was made in the hope of confirming some of Dunham and Baldwin's results and of testing the applicability of some of the explanations given above. Dwarf peas were grown in glazed earthenware pots each containing 3 kg. of nitrogen-deficient silver sand and supplied with the same nutrient solution as was used in the first two experiments. The sand was rendered free from nodule bacteria by blowing steam for 30 min. through a hole in the base of the pot. The nutrient solution was sterilized and watering was carried out with boiled rain water.

Two strains of nodule bacteria were used in the experiment, the effective strain Wisconsin 310, used by Dunham and Baldwin in their experiment, and the ineffective strain B 33, also obtained from Wisconsin.*

Set 1 received an inoculum of strain B 33 at the time of sowing, and was given a further heavy dose of the same strain when the plants were 6 weeks old. In set 3 the same strain was applied only after the peas had grown for 6 weeks without nodule bacteria.

Sets 4 and 6 were similarly inoculated with strain 310: set 4 receiving the bacteria both at sowing time and after 6 weeks, set 6 only after 6 weeks.

Set 2 received strain B 33 at sowing time and strain 310 after 6 weeks. Set 5 received strain 310 at sowing time and strain B 33 after 6 weeks.

Sets 7 and 8 received an inoculation of both strains applied simultaneously, suspensions of each strain containing approximately equal numbers of bacteria being mixed before application to the sand. In set 7 this mixture was applied both at sowing time and after 6 weeks and in set 8 only after 6 weeks.

An uninoculated control set was also included. The peas in this set remained free from nodules.

The inocula were applied in the food solution. The pots were allowed to dry somewhat before adding the second inoculation, to ensure that it mixed well with the sand. Each set consisted of four replicate pots except sets 1 and 4 which consisted of six pots each, from two of which the plants were removed after 6 weeks and their nodules counted. Four peas were grown in each pot from externally sterilized seed. After 16 weeks' growth, the remaining plants were removed from the pots and their nodules counted.

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* The authors' thanks are due to the Staff of the Wisconsin Agricultural Experiment Station for their kindness in supplying cultures of the strains of pea and soy bean *Rhizobium* used in this work and also of strains 202 and 205, isolated from clover.

Cultures were isolated from sixty nodules, fifteen taken at random from each pot, in each set that had received both strains. The cultures thus isolated were identified as belonging to strain 310 or B 33 according to the appearance of their growth on yeast agar slopes, which is very distinct as between the two strains. From these identifications the percentage of nodules belonging to each strain were estimated.

TABLE 3. EXPERIMENT 3. PEAS GROWN IN SAND

	strains supplied		mean number of nodules per pot				percentage containing	
set	at sowing	after 6 weeks	after 6 weeks	standard errors	after 16 weeks	standard errors	strain B 33	strain 310
1	B 33	B 33	550	± 32·1	548	± 25·0	—	—
2	B 33	310	—	—	473	± 25·8	96·1	3·9
3	—	B 33	—	—	266	± 43·0	—	—
4	310	310	332	± 51·5	417	± 42·0	—	—
5	310	B 33	—	—	417	± 9·8	11·6	88·4
6	—	310	—	—	306	± 39·0	—	—
7	B 33 + 310	B 33 + 310	—	—	434	± 10·0	95·5	4·5
8	—	B 33 + 310	—	—	285	± 5·5	94·6	5·4

The results of the experiment are set out in table 3. Sets 3 and 6, which were not inoculated until the plants were 6 weeks old, subsequently developed a mean of 266 and 306 nodules per pot respectively, thus showing that peas of this age were not too old to develop plenty of nodules.

Nodules produced during the first 6 weeks reduced the subsequent nodule formation from a re-inoculation made at this time. But this inhibition affected further nodule formation by the same strain as much as by a different strain. Thus strain B 33 produced 550 nodules per pot in the first 6 weeks (set 1) and the numbers found after, at 16th week, were not increased either by re-inoculation with the same strain (set 1) or by the other strain (set 2), though in the latter set a few nodules were produced by the second applied strain. Strain 310 produced 332 nodules per pot in the first 6 weeks. Re-inoculation with the same strain increased the mean number of nodules per pot by a further 85, developed between the 6th and the 16th week, resulting in a total of 417 nodules (set 4). But plants that bore no nodules during the first 6 weeks and were then supplied with strain 310, developed 306 nodules by the 16th week (set 6). Hence the presence of nodules during the first 6 weeks reduced the number of nodules subsequently produced from 306 to 85. The nodules formed during the first 6 weeks by strain 310 had a similar effect in reducing subsequent nodule formation in plants re-inoculated with strain B 33 (set 5). The root system of the plant thus has only a limited capacity for producing nodules: this limit was reached within the first 6 weeks by plants supplied with strain B 33 but was not attained during this initial period by plants supplied with strain 310 (sets 1 and 4).

The final nodules produced in set 5 show an increase of 85 on the 332 nodules produced during the first 6 weeks by strain 310. This figure represents about 20 % of the total 417 nodules produced during the whole growth period. Since 11·6 % of this total consisted of B 33 nodules, about half the nodules produced after the application of strain B 33 were due to this strain. Thus the experiment provides no evidence that the first-applied strain confers any specific immunity against subsequent nodule formation by a different strain. For, when strain B 33 was first applied (sets 1 and 2), the nodules formed during the first 6 weeks prevented any further increase in nodule numbers by either strain, while the lesser number of nodules produced by strain 310 within the first 6 weeks allowed an equal chance for further nodules to be produced by bacteria of strain 310, still present in the sand, or by those of strain B 33, subsequently added (set 5).

When the two strains were applied simultaneously in about equal numbers (sets 7 and 8) the ineffective strain B 33 dominated the nodule formation. This dominant strain also differed from strain 310 in that, when applied alone at sowing time, it enabled the plant to reach the limit of its nodule-producing power within the first 6 weeks (compare sets 1 and 4).

Experiment 4

Another pot experiment with peas was made to test the dominance of one strain over another when both were simultaneously applied. In this experiment, one set of four replicate pots was supplied with a mixed culture in about equal numbers of strains 310 and B 33, and another set with a mixture of strains 310 and 313, the latter being the ineffective strain which, together with strain 310, was employed in Dunham and Baldwin's work. The technique of this experiment was similar to the one above described. The plants were grown from 8 March till 12 June, when the nodules were counted and isolations of the bacteria were made from forty nodules of each set (ten per pot). The set given a mixed inoculum of strain 310 and B 33 produced a mean of 685 nodules per pot, 93 % of those tested containing strain B 33. The set given the mixture of strains 310 and 313 produced 703 nodules, strain 313 contributing 90 % of those tested. In these experiments with peas, two strains, both ineffective, in nitrogen fixation, were strikingly dominant as regards nodule production over the effective strain 310, when applied at the same time as the latter.

Experiment 5

An experiment on lines similar to experiment 3 was carried out with soy beans, which were grown in glazed earthenware pots each containing 12 kg. of nitrogen-deficient sand and supplied with 1 l. per pot of the same nutrient solution used in the previous experiments. The sand and nutrient solution were not sterilized, as it had been found that bacteria capable of producing nodules on soy beans were not naturally present in either. Two strains of nodule bacteria, both obtained from Wisconsin Agricultural Experiment Station, were used in this work; strain 501—an effective strain also used by Dunham and Baldwin (1931)—and the ineffective strain 507. The plant of the experiment was similar to that of experiment 3 except that the re-inoculations were carried out after 9 weeks' growth and that four instead of two additional pots inoculated at sowing time with each strain, were set up for the purpose of counting the nodules found at the time of the second inoculation. The remaining pots were kept on until the 16th week, when the nodules were counted and examined.

The nodules produced by the two bacterial strains 501 and 507 possess the fortunate character of being readily distinguishable from free-hand sections. Those produced by the effective strain 501 are soft in the centre, which is either dark red or olive green in colour; those produced by the ineffective strain 507 are hard in the centre, which is either white or very pale clear green. These differences are due to the different proportions of cells containing bacteria and of sterile starch-filled cells in the central tissue of the nodule (see Chen and Thornton 1940). Two hundred nodules produced by each strain in pure culture were cut across and every nodule could be correctly ascribed by this test to the strain which produced it.

At the conclusion of the experiment nodules were counted and about 100 nodules per pot were identified by free-hand sections. From these identifications the percentage of nodules produced by each strain was calculated for each of the sets with mixed inoculations. The results of this experiment are shown in table 4.

In this experiment, as in the last, the nodules produced on the young plants tended to inhibit the further formation of nodules by either strain. Strain 501 produced 247 nodules per pot by the 9th week; and re-inoculation by either strain at this time failed to cause any significant increase in nodule numbers by the 16th week (sets 1 and 2), although plants whose roots were nodule-free up to the 9th week and which were then supplied with strain 501 developed 229 nodules (set 3), and similar plants supplied at this time with strain 507 produced 159 nodules between the 9th and the

16th week (set 6). Strain 507 produced 129 nodules by the 9th week: after re-inoculation with the same strain, plants in set 4 finally produced 191 nodules, an increase of 62 nodules developed between the 9th and the 16th week. The difference between this increase and the 159 nodules produced by this strain during the same period on sterile roots in set 6 is 97 ± 27·18 and is quite significant.

TABLE 4. EXPERIMENT 5. SOY BEANS GROWN IN SAND

	strains supplied		mean numbers of nodules per pot				percentage containing	
set	at sowing	after 9 weeks	after 9 weeks	standard errors	after 16 weeks	standard errors	strain 501	strain 507
1	501	501	247	± 3·59	254	± 5·6	—	—
2	501	507	—	—	239	± 19·3	100	0
3	—	501	—	—	229	± 14·3	—	—
4	507	507	129	± 6·10	191	± 8·5	—	—
5	507	501	—	—	267	± 17·4	27	73
6	—	507	—	—	159	± 25·1	—	—
7	501 + 507	501 + 507	—	—	185	± 28·0	98	2
8	—	501 + 507	—	—	265	± 42·7	98	2

This experiment also failed to produce evidence that any greater immunity was conferred by the early nodules against a different strain subsequently applied, than against a further application of the same strain. In set 5, supplied with strain 507 at sowing time and with strain 501 after 9 weeks, the final nodule numbers, averaging 267 per pot, show an increase of 138 on the 129 nodules produced by strain 507 up to the time when strain 501 was applied. Thus, about 52 % of the nodules found at the end of the experiment were produced after the second inoculation. Since 27 % of the total 267 nodules were of the 501 type, this strain must have contributed about half of the nodules produced since it was applied. So that in set 2, where strain 501 was first applied, the nodules produced in the first 9 weeks prevented further nodule production by either strain, while in set 5, where strain 507, applied at sowing time, did not reach the limit of nodule production by the 9th week, the addition of strain 501 to the sand which still contained the first-applied strain 507, allowed each strain an equal chance to produce nodules.

When the two strains were applied simultaneously in about equal numbers (sets 7 and 8), one strain—in this case the effective strain 501—was dominant and produced 98 % of the nodules. The dominant strain again differed from the other in that, when applied alone at sowing time, it satisfied the nodule-producing power of the plant by the time of the second application of bacteria.

The conclusions derivable from these last two experiments are thus very consistent. In both of them one strain dominated the other when both were simultaneously applied. In both, the dominant strain, when applied at sowing time, had almost or quite satisfied the nodule-producing capacity of the plant before the time of the second application of culture, so that neither strain could produce further nodules. But when the dominant strain was applied second, to plants the limit of whose nodule-producing power was not reached, both strains had an equal opportunity to produce the remaining nodules.

The above experiments enable us to eliminate some of the possible explanations of Dunham and Baldwin's results, that were suggested and lettered above (p. 37):

(*a*) In our experiments, peas and soy beans first inoculated after 6 and 9 weeks respectively, produced plenty of nodules. The failure of the second-applied strain to produce nodules thus cannot be due merely to the age of the plant, either in our experiments or, by inference, in the work of Dunham and Baldwin who applied the second strain to plants of about the same age.

(*b*) In our experiments the first-applied strain did not immunize the plant specifically against a different strain.

(*c*) The nodules developed on the young plant tended to inhibit further nodule formation, either by the same or by a different strain, because their numbers approached or even reached the limit set by the nodule-bearing capacity of the root system. This limit was sometimes reached by the time the second culture was applied and may thus explain the failure of Dunham and Baldwin's peas and soy beans to develop nodules from the second-applied strain.

(*d*) When two strains are applied simultaneously in equal numbers to the surroundings of the root, it seems most unlikely that the almost complete dominance of one of them, as found in experiments 3, 4 and 5, could have been due to the dominant strain saturating the root system with nodules before the other strain was able to infect the roots, unless competition between the strains outside the plant enabled the dominant strain initially to repress the other in the root surroundings.

The following experiment was made in order to search for such a competition in the sand surrounding the root system.

Experiment 6

For this experiment dwarf peas were used together with the effective strain 310 and the ineffective strain 313. Experiment 4 had shown that the latter strain was strongly dominant as regards nodule formation when both strains were simultaneously supplied to peas grown in sand. In the present experiment, dwarf peas were grown in quart milk bottles, each containing 800 g. of nitrogen-deficient sand mixed with 1 g. of precipitated chalk. The bottles of sand were stoppered with cotton-wool and sterilized in the autoclave for 1 hr. at 15 lb. pressure. 100 ml. of the same food solution as was employed in the previous experiments were added to each bottle. Twelve replicate bottles were supplied with a pure suspension of strain 310 and twelve with a pure suspension of strain 313, each strain at a concentration giving about 1,000,000 bacterial cells per gram of sand. Twenty-four bottles were supplied with a mixed suspension giving about 1,000,000 bacteria of each of the two strains per g. of sand.

The food solution was autoclaved and the appropriate bacterial suspension mixed with it before adding it to the bottles.

All these bottles were sown with peas at the rate of six per bottle. The seed was sterilized externally by immersion in absolute alcohol for 3 min. and in a 0·2 % aqueous solution of $HgCl_2$ for a further 3 min. and washed in four changes of sterile water.

In addition to the above sets eight bottles were given a mixed inoculum of the two strains as already described but were not sown with peas.

The experiment was set up on 28 July and plate counts of the bacteria in the sand were made from the bottles sown with peas at the beginning and after 7, 16, 22, 29 and 60 days, and from the bottles without peas after 7, 16, 22 and 60 days. On each occasion duplicate bottles were sampled from the sets given pure cultures and from those without peas, and four replicate bottles from the set given a mixed inoculum and sown with peas. About 50 g. of sand were removed from each bottle into a sterile petri dish and well mixed with a flamed spatula. From this platings at several dilutions were made on yeast agar.[*]

The surface colonies of strains 310 and 313 are distinguishable, the former producing opaque white and the latter thin watery colonies. It was there-

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[*] The medium used for plate counts had the following composition:

K_2HPO_4	0·5 g.	Yeast water (15 % yeast)	100 ml.
$MgSO_4 . 7H_2O$	0·2 g.	Tap water	900 ml.
NaCl	0·2 g.		
$CaCO_3$	3·0 g.		

fore possible to make separate counts of the surface colonies of each strain in platings of a mixed culture. In order to make this separation possible, counts of colonies in all sets were limited to surface colonies. The bacterial numbers calculated were therefore too low but relatively correct.

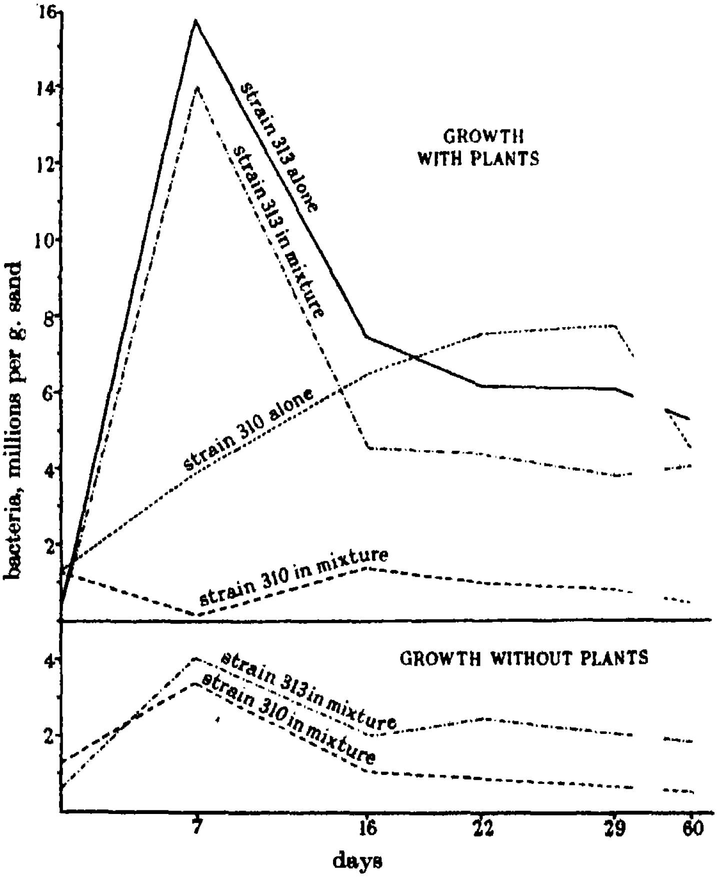

FIGURE 1. Growth of pea nodule bacteria in sand with and without plants (experiment 6).

The results of this experiment are graphically shown in figure 1, in which counts from each strain growing alone and in mixture with the other, are separately plotted. In the sets containing growing peas, counts made after 7 days showed a large rise in the numbers of strain 313 both in pure and in mixed culture. The numbers had fallen by the 16th day after which they remained fairly steady. The presence of strain 310 slightly decreased the numbers of strain 313 as compared with those reached by the latter in pure culture. In the presence of the plants, strain 310 in pure culture grew at first much more slowly but showed a steady increase up to the 29th day. But in mixed culture the increase of strain 310 was entirely prevented by competition with the faster-growing dominant strain 313. The percentage of the mixture composed of strain 313 varied at different times, but the mean percentage over the run of the experiment was 88·4. The proportion of the two strains in the sand in the presence of the plant is therefore in good agreement with that of the nodules developed by each from a mixture of these two strains applied to the plant in experiment 4, where 90 % of the nodules contained strain 313. This suggests that the two strains, when in contact with the root, have an equal chance of infecting the plant, the relative number of nodules developed by each strain being an expression of the proportions of the two strains in the sand surrounding the roots, a proportion resulting from competition between them.

The complete suppression of growth of strain 310 by the dominant strain 313 would seem to be related to the early rapid growth of the latter strain. Hence when a normally dominant strain is applied some weeks after the other strain to root surroundings already populated by the latter, the dominance should not show itself. This conclusion was borne out by experiments 3 and 5. In set 5 of experiment 3 strain 310 was applied at sowing time and strain B 33 after 6 weeks. The two strains contributed about equally in producing the nodules that subsequently developed; although strain B 33 is strikingly dominant when applied simultaneously with strain 310 (sets 7 and 8, experiment 3). A similar phenomenon appears in the soy bean (experiment 5).

The early rapid growth of the dominant strain also offers an explanation for the early saturation of the nodule-producing power of the plant by dominant strains when applied at sowing time; this holds for both peas (experiment 3) and soy beans (experiment 5).

In the set without peas in the present experiment the number of bacteria of either strain did not rise above the 4 millions per g. of sand at any time of sampling and there was in consequence no evidence of any competition between the strains. The rapid increase in strain 313, and its inhibiting effect on strain 310, are thus related to the presence of the plant's roots, although taking place outside them. The experiment indicates that the roots secrete an energy source or an accessory growth substance that stimulates the growth of both strains but especially that of strain 313. An experiment was therefore made to test the effect of replacing the root secretions by a pure carbohydrate as energy source.

Experiment 7

The same method was, in general, followed in this as in the last experiment, save that no peas were grown in any set. Twenty-eight bottles received the basal food solution without carbohydrate and twenty-eight a further addition of 0·2% sucrose. Eight bottles of each medium were given a suspension of strain 310, eight bottles received a suspension of strain 313 and the remaining twelve bottles a mixture of the two strains. The cultures were applied at a concentration giving about one million bacteria of each strain per g. of sand. Plate counts of the two strains were made at the start and after 4, 6 and 13 days using the method described for experiment 6. Counts of the pure cultures were made on each occasion from duplicate bottles and of the mixed cultures from triplicate bottles. The results are shown in figure 2.

In the bottles without either plants or added energy supply, the number of cells of either strain, alone or in mixture, did not rise above 5·1 millions per g. and there was no evidence of competition between the two strains. This is in agreement with the behaviour of the similar sets in the last experiment. In the presence of sugar, strain 313 when in pure culture rose to 28 millions in 4 days, after which its numbers fell to 17·3 millions by the 13th day. In the presence of the other strain the numbers of strain 313 rose to 19·1 millions by the 4th day. Strain 310 when in pure culture grew much more slowly, reaching only 10·1 millions by the 4th day, and thereafter rising steadily to 27·5 millions by the 13th day. In mixture with the dominant strain 313, the growth of strain 310 was again severely checked, the numbers of this strain never rising above 3·6 millions.

The course of growth of the two strains in this set with added dextrose closely resembles that found in the last experiment when pea roots were growing in the sand. We have in both cases the same rapid rise in numbers of the dominant strain whether grown alone or in mixture, and the slower growth of strain 310, which is almost inhibited by the presence of strain 313.

The experiments described above suggest the following explanation of the behaviour of two strains, one dominant over the other, as exemplified by the pea nodule strains here studied.

The plant's root system secretes substances that stimulate the multiplication of nodule bacteria in the root surroundings, but the rate of multiplication thus induced differs greatly with different strains. A strain which at first multiplies rapidly under these conditions will quickly satisfy the nodule-producing power of the plant and prevent the further nodules being formed either by itself or by any other strain supplied later.

When a strain that multiplies more slowly at first is applied in pure culture at sowing time, it will take longer to reach the limit of nodule production, so that further nodules may be produced by a different strain later applied.

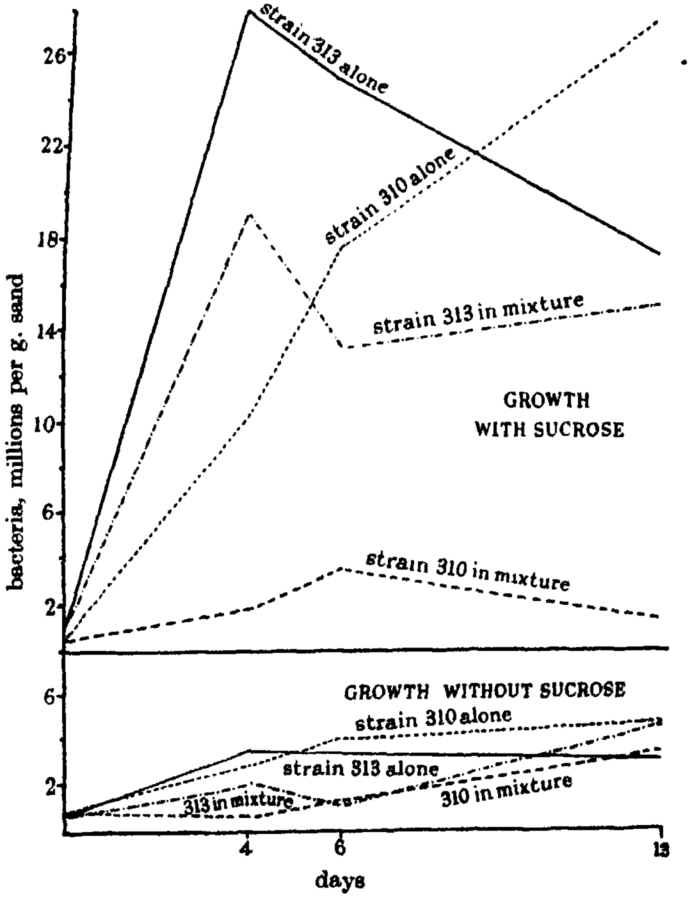

Figure 2. Growth of pea nodule bacteria in sand without plants (experiment 7).

When two strains with different multiplication rates are supplied together, the strain that multiplies more rapidly at first will compete with, and suppress the growth of, the other strain, and will largely prevent it from forming nodules.

3. FACTORS AFFECTING THE COMPETITION FOR NODULE PRODUCTION BETWEEN STRAINS OF CLOVER *RHIZOBIUM*

In the experiments with peas and soy beans, a limit to the nodule-bearing power of the root at a fairly young stage of the plant was an important factor, which was sufficient to explain the high percentage of nodules produced by the first-applied strain in sets receiving successive inoculation with two strains, and which must have increased the importance of early competition outside the roots between two strains, both supplied together at sowing time.

This early limitation would seem to be connected with the short life of the pea and soy bean, whose root systems make a large part of their growth within the first 6 weeks. In clover, the root system grows comparatively slowly at first but continues its growth for a long period. In this plant therefore no early limitation to the number of nodules is to be expected.

The following experiment was made in 1939 to determine whether, in the clover plant, the first-formed nodules exerted any inhibiting effect on the further production of nodules by the same or by a different strain.

Experiment 8

Red clover was sown in quart milk bottles each containing sand and 100 ml. of the following food solution:

K_2HPO_4	1·0 g.	$CaCl_2$	0·1 g.
$MgSO_4 . 7H_2O$	0·2 g.	$FeCl_3$	0·02 g.
NaCl	0·1 g.	Water	1 l.

Six replicate bottles were inoculated with strain A, six with the Coryn strain and eight were left uninoculated. The seed was sown on 26 July and on 7 September the plants were removed, their roots washed in sterile water and the nodules counted. The sand from the bottles supplied with the two strains was placed in separate earthenware pans, four pans containing sand infected with each strain. The seedlings were then replanted in these pans so that each pan contained two plants bearing nodules produced by each strain, and two plants without nodules at the time of transplanting. After 2 months' further growth the plants were removed and their roots examined. Some of the plants died, the numbers that survived being shown in table 5. The check due to planting out had produced a local thickening on each root, which made it easy to determine what portion had grown since then. This callus formation enabled a distinction to be drawn between the nodules formed on the old roots, and those produced on the new roots and hence undoubtedly due to fresh infections from the sand. The results are shown in table 5.

Table 5. Experiment 8. Nodule development on transplanted clover

set	no. of plants	original nodules strain	mean no. of nodules when trans-planted	standard errors	final nodule numbers	standard errors	increase	no. of nodules on new roots	standard errors
Transplanted into sand containing the Coryn strain									
1	4	A	14	± 2·6	90	± 15·2	76	74	± 16·6
2	9	Coryn	34	± 3·1	118	± 10·1	84	76	± 11·3
3	5	—	—	—	71	± 13·6	71	71	± 13·6
Transplanted into sand containing strain A									
4	10	A	23	± 3·8	55	± 7·0	32	30	± 5·7
5	4	Coryn	32	± 3·7	78	± 12·7	46	43	± 10·4
6	6	—	—	—	46	± 8·9	46	46	± 8·9

The number of nodules produced on the new roots has been quite unaffected by the presence of nodules at the time of transplanting. These first-formed nodules have not checked the further development of nodules either by the same or a different strain. There is, on the other hand, a characteristic difference in the number of nodules produced on the new roots by the two strains. The plants growing in pans containing Coryn bacteria developed a mean of 73·4 nodules per plant on the new roots, those in pans containing strain 205, a mean of only 39·9 nodules.

There are, indeed, great differences in the rates of nodule production on clover by different bacterial strains. Three strains were used in the next part of the work, namely, the effective strains 205 and A and the ineffective Coryn strain. The rates of nodule production on red clover seedlings grown on agar slopes* and supplied with each of these three strains are shown graphically in figure 3. Each strain gives a characteristic curve. Coryn produces a few nodules between the 10th and 20th day and thereafter there is a rapid increase in the number of nodules produced. Strain A begins to produce a few nodules at the same time as Coryn but nodule production thereafter is much slower. Strain 205 does not produce its first nodules until about 10 days after the other two strains have done so, but later the number of nodules formed by this strain increases at about the same rate as do those of strain A. Since in clover the number of nodules is not determined by the establishment of a limit to further infection in the early stages of the plants' growth, their number will be dependent on the specific infectivity of the strain forming them.* Hence, when equal numbers of two strains are simultaneously applied to the root surroundings, the number of nodules due to each strain in the mixture should be proportionate to the numbers produced by each strain in pure culture, unless some other factor such as competition outside the root interferes.

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* The composition of this agar medium was similar to that used in experiment 9 (p. 52).

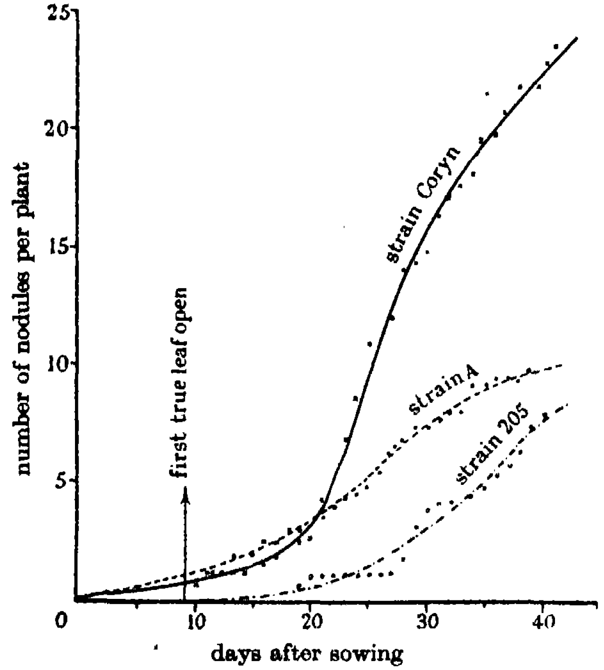

FIGURE 3. Rate of appearance of clover nodules in agar culture.

The following experiment was made to measure the proportion of nodules produced by each strain when bacteria of two strains were simultaneously applied to the root surroundings in about equal numbers. Two such pairs of strains were tested, namely, Coryn with 205 and Coryn with A. These two pairs were chosen because experiment 2 had shown that the ineffective strain Coryn depressed the growth and nitrogen fixation otherwise produced by strain 205 but was without effect on the action of strain A.

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* A further investigation of specific infectivity is described by Chen (1941).

Experiment 9

Red clover was grown in quart milk bottles containing 800 g. of nitrogen-deficient sand and 100 ml. of the following food solution:

K_2SO_4	8·75 g.	$MgSO_4.7H_2O$	5·0 g.
$CaSO_4.2H_2O$	3·0 g	NaCl	5·0 g.
K_2HPO_4	2·75 g.	$FeCl_3$	0·02 g.
KH_2PO_4	3·0 g.	Water	1 l.

The bottles with sand were sterilized for 1 hr. and the food solution for 15 min. at 15 lb. pressure before addition to the bottles. The bottles were inoculated according to the following plan, the bacterial suspension being added to the food solution in such a manner that all sets were given about equal total numbers of bacteria and those treated with a mixed inoculum received about equal numbers of each strain.

set	inoculation	no. of replicate bottles
1	strain 205	16
2	strain 205 + Coryn	8
3	Coryn	12
4	A	24
5	A + Coryn	12

About fifteen seeds, externally sterilized with absolute alcohol and $HgCl_2$, were sown in each bottle on the surface of the sand. After 3 months' growth the clover roots were washed, the nodules counted and the lengths of a number of nodules, varying from 30 to 100 per bottle according to the strain, were measured. In the sets given double inoculations the percentages of nodules containing each strain were estimated from tests made on cultures isolated from nodules taken at random, and these estimates were checked by the frequency distribution of nodule lengths, as described below. The experiment falls naturally into two parts, that dealing with the interaction of strains Coryn and 205 and that dealing with the interaction of Coryn with strain A. These will be separately considered.

The Coryn strain is difficult to distinguish in vitro from strain 205 owing to its close similarity in growth and in physiology. It can, however, be distinguished in the plant by three characters: (*a*) nodule formation on the seedling grown in agar commences about 10 days later with strain 205 than with the Coryn strain (see figure 3); (*b*) 205 nodules, when they reach a certain size, are usually of a distinct pink shade; (*c*) Coryn nodules have a much smaller mean length than those produced by strain 205.

Cultures were isolated from 130 nodules (about 16 per bottle) taken at random from plants of set 2, that were given the mixed inoculum of strains 205 and Coryn. These were supplied to red clover seedlings grown in an agar medium having the following composition:

K_2HPO_4	0·5 g.	$Ca_3(PO_4)_2$	2·0 g.
$MgSO_4.7H_2O$	0·2 g.	$FeCl_3$	0·01 g.
NaCl	0·1 g.	Agar	10·0 g.
$FePO_4$	1·0 g.	Water	1 l.

The time of appearance of the first nodule was noted and, after further growth, the presence or absence of pink coloration in the larger nodules was noted. These two tests were in good agreement and indicated that 89·3 % of the cultures were of the Coryn strain.

The number of nodules per plant grown in the milk bottles, and their mean lengths, are shown in table 6. The nodule lengths provide a check on the estimate of the composition of the mixed set formed from the isolations. The mean length of Coryn nodules on plants of set 3 supplied with a pure culture of the strain was 0·46 mm.; that for strain 205 in set 1 was 1·13 mm. If 89·3 % of the nodules in the set with the mixed culture had the former mean length and 10·7 % had the latter, the mean length of the nodules in the set should be 0·53. It was actually 0·53 in set 2. A finer check can be obtained from the frequency distribution of nodule lengths in the three sets. Nodules produced by pure cultures of 205 and Coryn have very distinct frequency distributions of length (figure 4). The frequency distribution of nodule lengths to be expected in the mixed culture can be deduced by calculating, for each nodule length, the term $(f_c c + f_a a)/100$, where f_a and f_c are the frequencies of that particular length for strain 205 and Coryn respectively when supplied in pure culture, and

TABLE 6. EXPERIMENT 9. CLOVER GROWN IN QUART BOTTLES. INTERACTION OF STRAINS 205 AND CORYN

set	no. of plants	strains supplied	mean nodule numbers per plant	standard errors	percentage of Coryn nodules estimated from isolations	mean nodule lengths in mm.	standard errors	no. of nodules measured
1	184	205	16	± 3·4	—	1·13	± 0·025	534
2	115	205 + Coryn	56	± 5·3	89·3	0·53	± 0·01	564
3	146	Coryn	64	± 0·8	—	0·46	± 0·02	840

where a and c are the percentages of strains 205 and Coryn estimated from the isolations. The lower diagram in figure 4 shows the expected frequency curve obtained by this calculation together with the distribution actually observed. Both these curves closely resemble that derived from the pure Coryn strain.

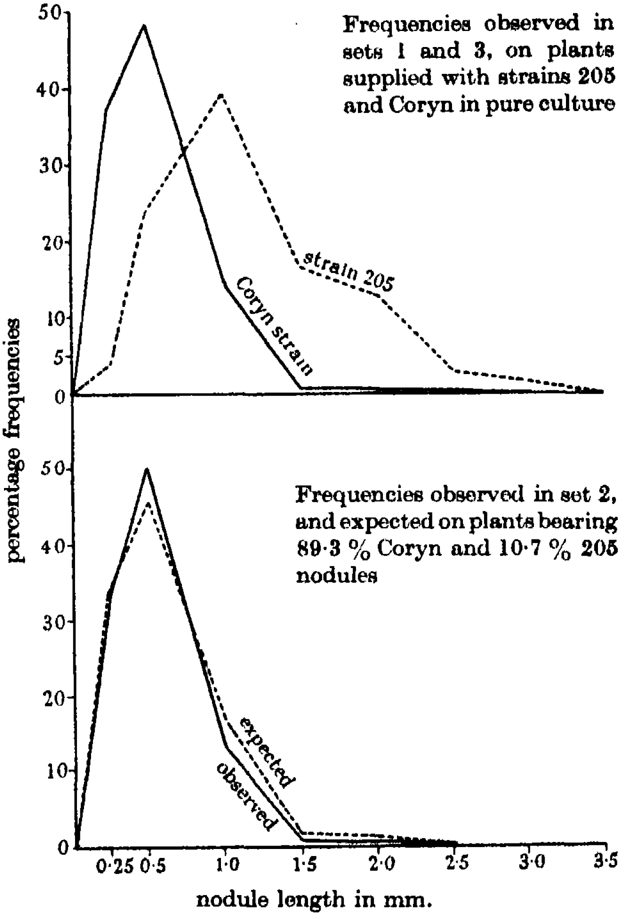

Figure 4. Frequency distributions of nodule length in experiment 9, sets 1, 2 and 3.

The large percentage of Coryn nodules in the set supplied with a mixture in about equal numbers of the two strains can largely be accounted for by the very different nodule numbers characteristic of each strain in pure culture, and due to the different degrees of infectivity. Strain 205 by itself produced 16 nodules per plant in set 1 while the Coryn strain produced 64 in set 3. The figures are in a ratio of 1 : 4. If each strain in the mixture had an equal opportunity to produce its characteristic degree of infection, the numbers of nodules produced by the two strains should be in this proportion, which would give 80 % Coryn nodules in the mixture. The difference between this figure and the percentage 89·3 actually found in set 2 leaves very little to be accounted for by selective competition between the strains either outside or within the plant.

The data obtained with strains A and Coryn are set out in table 7.

TABLE 7. EXPERIMENT 9. CLOVER GROWN IN QUART BOTTLES. INTERACTION OF STRAINS A AND CORYN

set	no. of plants	strains supplied	mean nodule numbers per plant	standard errors	percentage of Coryn nodules estimated from isolations	mean nodule lengths (mm.)	standard errors	no. of nodules measured
4	252	A	11	± 0·6	—	1·09	± 0·01	714
5	87	A + Coryn	17	± 2·0	7·9	1·075	± 0·03	278
3	146	Coryn	64	± 0·8	—	0·46	± 0·02	840

The nodules produced by these two strains are distinguishable by the following characters. (*a*) Strain A seldom produces more than ten nodules per plant on red clover seedlings grown for 1 month in agar, while Coryn seldom produces less than fifteen. (*b*) The nodules of strain A more than 1 mm. long are nearly always coloured pink. (*c*) There is again a marked difference in mean nodule length between the two strains.

Cultures were isolated from 140 nodules taken at random from the set given a mixed inoculum of strains A and Coryn. These cultures were tested on red clover seedlings grown in agar medium. After 1 month's growth the nodules were counted and the isolations were classified according to the number of nodules per plant and the presence or absence of pink pigment. These tests indicated that 92·1 % of the nodules tested contained strain A. This estimate can again be checked by the lengths of nodules in the bottles (see table 7). The mean nodule lengths found in bottles supplied with a pure culture of strains A and Coryn was 1·09 and 0·46 respectively (sets 3 and 4). Hence a mixture of the two strains containing 92·1 % of strain A nodules should have a mean length of 1·043 mm. The figure actually obtained from 278 nodules taken from the bottles in set 5 given the mixed inoculum was 1·075 mm. The frequency distributions of nodule lengths of Coryn and strain A are again characteristic. They are shown in figure 5.

From these can be calculated the distribution of nodule lengths expected in a mixture of 92·1 % A and 7·9 % Coryn nodules. This expected distribution and that actually obtained from the mixed set are compared in the lower diagram in this figure.

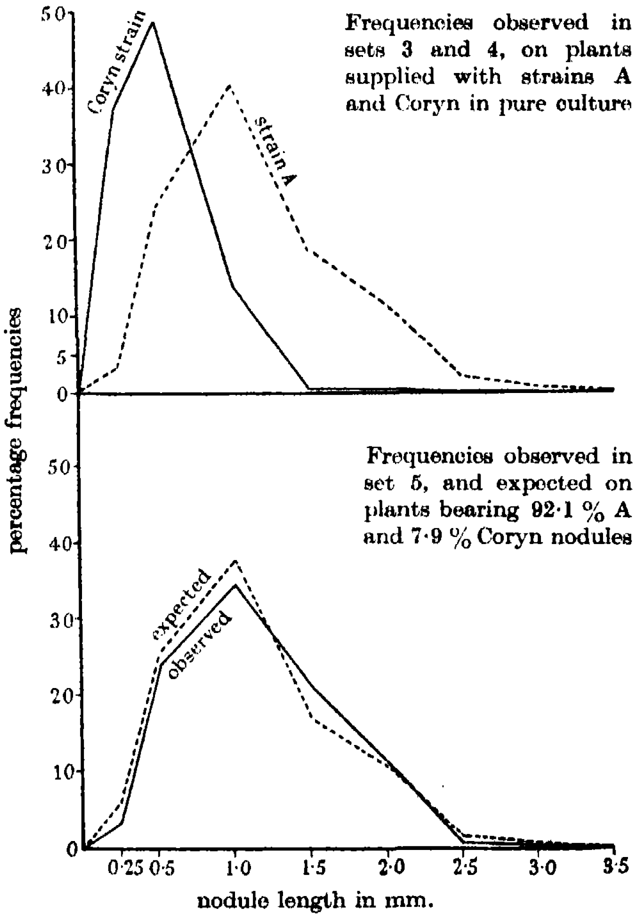

FIGURE 5. Frequency distributions of nodule lengths in experiment 9, sets 3, 4 and 5.

The interaction of strain A with the Coryn strain is in marked contrast with that of strain 205. The mean number of nodules per plant produced by strain A in pure culture was 11 (set 4), while Coryn produced 64 nodules (set 3). These figures are in the ratio of 1 : 5·68, so that if the strains did not interfere with each other but each had an equal chance to produce its characteristic degree of infection, a mixture in equal numbers of the two strains, as was supplied in set 5, should bear nodules of which 85% should be produced by the Coryn strain. In fact only 7·9% were found to contain Coryn. That this is due to an inhibition of the Coryn strain and not to a stimulation of nodule production by strain A is shown by the small number of nodules per plant (17) produced in the mixed set 5. This is only slightly higher than the number (11) produced by strain A in pure culture.

In experiment 8, plants bearing strain A nodules and transplanted, after washing their roots, into sand containing only Coryn bacteria, subsequently produced as many nodules as did plants that were without nodules at the time of transplanting. Hence the repression of Coryn nodules by strain A in the present experiment cannot be due either to satisfaction of the nodule-bearing capacity of the plant by strain A or to the establishment by it of a specific immunity against infection by the Coryn strain. It can thus be accounted for only through competition between the two strains outside the plant. The different results of the two experiments are associated with the fact that in experiment 8 the two strains were applied in succession whereas in this experiment they were present simultaneously in the sand, initially in equal numbers, so that competition *outside* the plant could take place.

In the case of pea nodule bacteria the dominance in strain 313 in competition with strain 310 was attributed to the more rapid early growth of the former strain. It was therefore to be expected that strain A would also multiply more rapidly than the Coryn strain in the sand surrounding clover roots. The following experiment was made to see whether this was so.

Experiment 10

Pure cultures of strain A and of the Coryn strain were grown in quart milk bottles containing washed sand, using the same technique and the same food solution as in experiment 6. In making the inoculations, a suspension of each strain was counted on a haemocytometer and the two suspensions were standardized so as to contain equal numbers of bacteria. An equal volume of suspension was added to each bottle. Nine replicate bottles were supplied with each strain and all were sown with externally sterilized red clover seed at the rate of about twenty seeds per bottle, on 20 February.

Plate counts on yeast agar were made of the suspensions at the start and from samples of the sand taken from triplicate bottles, after 9, 14 and 18 days. The results are shown in figure 6. By the 9th day the clover seedlings had their first true leaves open and had begun to develop nodules. By this time the numbers of the dominant strain A in the sand were about double those of the Coryn strain. Thus the strain dominant as regards nodule formation is again, as in pea nodule bacteria, characterized by a more rapid multiplication in the root surroundings than the strain against which it competes with success.

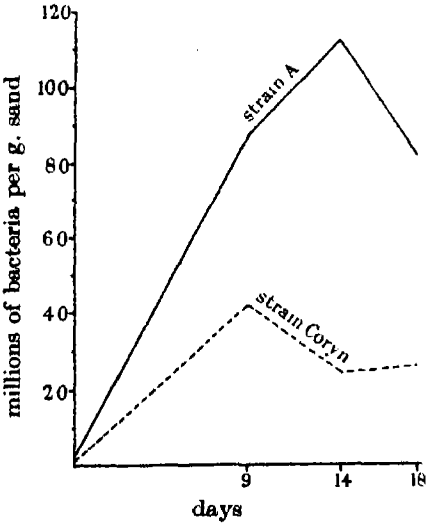

FIGURE 6. Growth of strains A and Coryn in sand surrounding clover roots (experiment 10).

CONCLUSION

The effect of dominance in competition outside the root system would seem to be of paramount importance in determining which of two strains shall contribute most to the production of nodules when both are present in the surroundings of their host root system. This competition, when it occurs, masks the influence of the relative infectivity of the two strains, and shows its effect regardless of whether the first-formed nodules inhibit later ones, since this inhibition is not selective in its action.

There is need for further research on the ecology of nodule bacteria in the surroundings of legume roots. The behaviour of mixed cultures of two or more strains in this environment affords an almost untouched field for work.* In preparing cultures for commercial distribution, it becomes of practical importance to choose strains that can not only produce nodules beneficial to their host plant, but are also dominant in competition with other strains. The association of this dominance with a high initial growth-rate in sand supplied with an energy source, suggests a convenient laboratory method for assessing the ability of a strain to compete with others.

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* Amongst the points that need investigation is the influence of bacteriophage on strain competition. The cultures used in the present work were tested for bacteriophage with negative results. But the equilibrium between strains growing in mixture might well be disturbed by the presence of a bacteriophage capable of attacking one of them.

The intense competition that takes place between closely related strains of bacteria may well have a wider importance, in its application to pathogenic organisms. The work of Greenwood, Bradford Hill, Topley and Wilson (1936) on mouse epidemics has emphasized the distinction between the infectivity and the virulence of pathogenic strains. In *Rhizobium*, Chen and Thornton (1940) have produced evidence that the quantity of nitrogen fixed by a nodule is a function of the growth of the bacteria within it. This is a strain character analogous to virulence in a pathogenic organism. Strains also differ specifically in the number of nodules that they produce, that is, in their infectivity. The present work introduces the further concept of dominance in competition between strains before infection occurs. This competition can be compared with the intra-specific selection that takes place in higher organisms and may well be more acute than competition between more distantly related micro-organisms whose environmental requirements are more dissimilar.

The authors wish to thank Dr H. K. Chen who carried out experiment 8 and who assisted in carrying out experiment 5.

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The reaction between type II pneumococcus antiserum and a glucuronide azo-protein

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An amino derivative of the natural glucuronide, euxanthic acid, was prepared and coupled by the azo-link to proteins.

These azo-proteins gave precipitates with type II pneumococcus antiserum, which were inhibited by glucuronides. The effect of varying azo-protein/serum ratio on the amount and composition of the precipitate was similar to the effect of varying polysaccharide/serum ratio on the precipitate formed when the specific polysaccharide is added to the serum.

Attempts to immunize rabbits and mice with amino-euxanthic acid azo-protein were not successful.

Chemical estimations and animal protection experiments indicated that the material precipitated by the azo-protein from the serum was part of the antibody to type II pneumococcus.

These results support the view that at any rate part of the specificity of type II serum for the type polysaccharide is due to glucuronic acid or some closely similar grouping.

Work with artificial antigens, notably the azo-protein studies of Landsteiner and his school, has established the following facts about the chemical specificity of immune reactions with this class of antigen:

(1) An animal immunised with a protein coupled with a well-defined chemical grouping gives an antiserum that will precipitate the original or any other protein coupled with this 'determinant group'.

(2) Smaller precipitates are sometimes given with proteins coupled to groupings closely related chemically to the determinant group.

(3) Small molecules containing the determinant group—'haptenes'—inhibit the formation of the precipitate. These facts are reasonably explained by the hypothesis that the antibody contains combining areas having a specific affinity for the determinant groups in the antigen, and sometimes a smaller affinity for chemically related groups; and that haptene molecules prevent access of the antigen by combining with and so blocking the receptive areas of the antibody.

The question arises, whether these artificial antigens are valid models of natural antigens. In other words, can immunity to unaltered proteins, natural pathogens and toxins, and the like, be traced to definite determinant groups in the antigen?

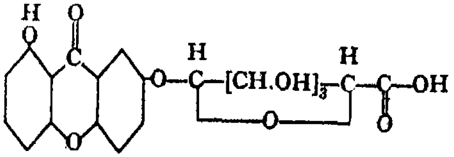
The possibility of answering this question for certain pathogens was opened up by Heidelberger and Avery, who isolated, from various serological types of the pneumococcus, complex bodies of polysaccharide nature, chemically different for each type, and giving specific precipitates with the type antiserum. Similar specific substances have been isolated from cultures of other organisms. The polysaccharide of type II pneumococcus has acidic properties, and on hydrolysis yields glucuronic acid. Heidelberger, Avery and Goebel (1929) showed that gum acacia, partially hydrolysed to remove part of the pentose, gives a precipitate with type II pneumococcus antiserum. This gum product also has acidic properties and gives glucuronic acid on hydrolysis. Marrack and Carpenter (1938) showed that a number of other partially hydrolysed plant gums, most of which have been shown to contain glucuronic acid, give similar precipitates. Precipitation of the glucuronic-acid-containing gums is inhibited by glucuronic acid and simple glucuronides, but not by other sugars or sugar acids. Glucuronic acid also delays the appearance and reduces the amount of precipitate from type II antiserum and the pneumococcus polysaccharide. These observations are similar to the results with artificial antigens, and strongly suggest that the specificity for type II pneumococcus antiserum of the plant gums is wholly, and of the pneumococcus polysaccharide at least partly, due to the presence in the molecule of the glucuronic acid residue or some closely related chemical grouping.

In order to test this suggestion, it was considered desirable to prepare an artificial antigen, having nothing in common with the pneumococcus polysaccharide except the glucuronic acid group, by coupling a suitable glucuronic acid compound to a protein, and to study the behaviour of this compound towards type II pneumococcus antiserum. The work to be described gives the results of such a study.

When the work was commenced, there was no method of preparing an artificial glucuronide suitable for coupling to a protein. Search was therefore made for a suitable compound among the natural glucuronides, and an ideal substance was found in euxanthic acid. Shortly after a preliminary account had appeared of the results with euxanthic acid (Woolf, Marrack and Downie 1936), a brief report was published by Goebel of results with a *p*-amino-phenol-glucuronide-azo-protein (Goebel 1936*a*), followed by a full account (Goebel 1936*b*). Goebel had succeeded in working out a general method for attaching uronic acid residues to proteins, and had shown that his glucuronide-azo-proteins reacted in high dilutions with antipneumococcus horse sera of types II, III and VIII. Thus, while the work described in this paper was being done, with amino-euxanthic acid azo-protein as test antigen, Goebel was simultaneously doing similar experiments with his *p*-amino-phenol-glucuronide-azo-protein. It is remarkable how closely the results agree. Inhibition by glucuronides but not by derivatives of other sugar acids was observed in both cases. In neither case was the glucuronide-azo-protein able to protect rabbits against pneumococcus infection. In the work described below, the presence of pneumococcus antibody in the azo-protein precipitate was proved by animal tests. Goebel and Hotchkiss (1937) obtained strong evidence by means of absorption experiments.

The results of Goebel and those to be described mutually confirm one another. It is worth while commenting on the different chemical methods used. The general method devised by Goebel is of course of the greatest importance, as enabling any uronic acid residue to be tested as required against a serum. But the methods described below may also be of value in future work. Suitable natural compounds may render unnecessary a tedious synthesis, or be used to confirm results obtained with synthetic material. The method of introducing the amino-group, by coupling with a diazo-compound and subsequent reduction, may also be of value even in synthetic procedure, as a possible alternative to the introduction and reduction of a nitro-group. As applied to the natural osides and uronides, it allows of any such compound with a free para- or ortho-position to a phenolic hydroxyl group being coupled to a protein, greatly extending the range of natural compounds that can be used. There is thus room for the application of the chemical methods here described as a useful supplement to the general synthetic procedure described by Goebel.

The formula of euxanthic acid is

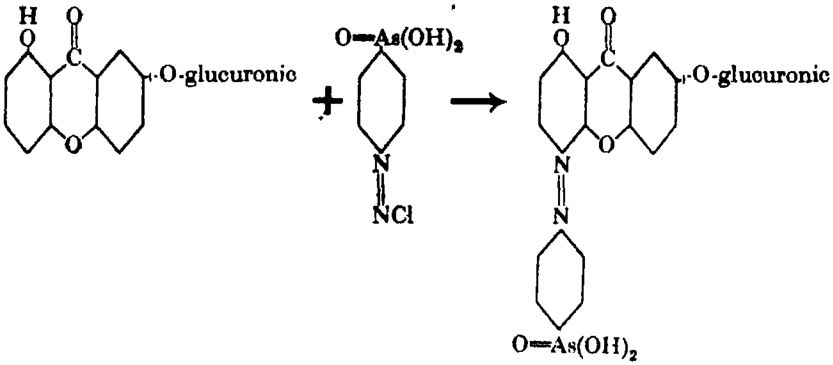


Unlike most glucuronides, it is highly stable, being hydrolysed only by boiling dilute acid under pressure. It is easily purified by recrystallization from alcohol. The benzene ring shown on the left contains a free place *para* to a phenolic hydroxyl group, where a diazo-compound can readily be coupled. The only disadvantage of euxanthic acid is its scarcity. All the work described was done with a few grams of the compound.

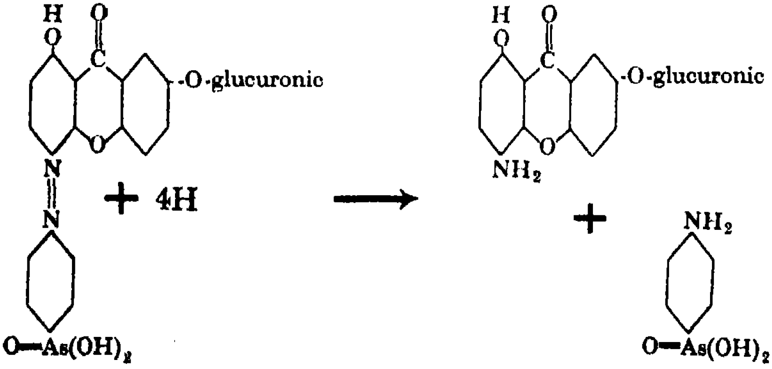
Preliminary tests showed that sodium euxanthate inhibited gum acacia precipitation and delayed the precipitation of the specific polysaccharide by type II antiserum in concentrations comparable with those of glucuronic acid itself, so that the glucuronic residue in euxanthic acid is in a suitable configuration for testing against the type II antiserum.

In order to couple the euxanthic acid to a protein, it is necessary to introduce an amino-group into the molecule. This was done by the following series of reactions:

(1) *Coupling:*



(2) *Reduction:*



A weighed amount of atoxyl (*p*-amino-benzene-arsinic acid) was diazotized and poured into an ice-cold solution of the stoichiometric quantity of euxanthic acid dissolved in excess of dilute caustic soda. The yellow solution rapidly turned deep red. After standing overnight, the atoxyl-azo-euxanthic acid was precipitated by hydrochloric acid, filtered off from the red mother liquor, and washed with dilute acid till the washings were colourless. It was then dissolved in dilute sodium hydroxide, brought to the boil, precipitated with hydrochloric acid, and allowed to cool. The compound was obtained as bright yellow needles. The atoxyl compound was used as intermediary because, of the compounds tried, it was the easiest to purify. It behaved like glucuronic acid in inhibiting precipitations by the type II antiserum.

Great difficulty was experienced at first in reducing the azo-compound to obtain the free amino-euxanthic acid. Most reducing agents gave brown products containing impurities which could not be eliminated. Finally, the following procedure was invariably found to give a crystalline product. The azo-compound was dissolved in a very small volume of dilute sodium hydroxide, and excess of solid sodium hydrosulphite was added. After half an hour, dilute hydrochloric acid was added drop by drop until a maximum precipitate was obtained. This point was very easy to observe, as the solution became almost solid with a feltwork of tiny crystals. After cooling to 0° C, the precipitate was filtered off, washed with a very little ice-cold water, and suspended in a small volume of water. It was then cautiously heated until solution was complete, filtered hot, and allowed to crystallize in the refrigerator. Recrystallization was carried out in the same manner. The amino-euxanthic acid was obtained as golden yellow long square prisms. It was easily soluble in acids and alkalis, and in warm water, and very sparingly soluble in ice-cold water.

Analysis:

	C %	H %	N Kjeldahl %
found	52·36	4·49	3·15
	52·15	4·30	3·24
			3·13
calculated for $C_{19}H_{17}O_{10}N, H_2O$	52·13	4·38	3·20

Titration with nitrous acid showed that all the nitrogen was present as an aromatic amino group.

Amino-euxanthic acid behaves like glucuronic acid in inhibiting precipitations by type II antiserum.

Amino-euxanthic acid azo-proteins were prepared as follows. A weighed amount of amino-euxanthic acid was dissolved in dilute hydrochloric acid, and diazotized at 0° C with sodium nitrite in the usual way. The diazo solution was poured into an ice-cold solution of the protein, enough sodium hyroxide being added to make the solution just alkaline. The colour gradually deepened. but complete coupling was rather slow, and the mixture was left overnight in the refrigerator. The azo-protein was precipitated by hydrochloric acid, centrifuged off, suspended in a little water, and redissolved by being rubbed up with a glass rod while sodium hydroxide solution was cautiously added, care being taken to avoid local excessive alkali concentrations that would give rise to insoluble lumps. The gelatinous mass was rubbed up with several volumes of water until solution was complete, and the azo-protein was again precipitated with acid and centrifuged off. The solution and precipitation were repeated until the supernatant was pale or colourless, usually after three or four treatments. The alkaline solution of azo-protein was then placed in a collodion sac, and dialysed in the refrigerator against several changes of 0·9 % sodium chloride solution, until coloured products ceased to dialyse out. Any precipitate which formed was centrifuged off. The azo-protein solution so obtained was a neutral, clear deep brown fluid, isotonic with serum. For immunizing rabbits, the protein used was horse serum globulin. For precipitating immune sera, the amino-euxanthic acid was coupled to twice recrystallized egg albumin. The ratio of protein to amino-euxanthic acid, in various preparations, ranged between 2 : 1 and 10 : 1.

The azo-protein readily gave precipitates with horse antiserum to type II pneumococcus, the effect being still observed when the azo-protein was diluted to 1 : 10,000. Precipitation was inhibited by glucuronic acid, euxanthic acid and its derivatives, and borneol glucuronide, but not by mannuronic or galacturonic acids. The precipitates were readily soluble in glucuronic acid and glucuronides, but not in the other uronic acids. They were also easily soluble in alkali.

The 'zone phenomenon' was observed. In a series of tubes containing a given volume of serum and increasing amounts of azo-protein, there was an 'optimum ratio' at which precipitation was observed to begin earliest. The more the ratio differed from the optimum, whether the serum or the azo-protein was in excess, the later the commencement of precipitation. Dilution of the mixtures with saline did not alter the optimum ratio. This was precisely analogous to the behaviour of the serum with the specific polysaccharide or the gums.

Figure 1 shows the effect of varying the amount of azo-protein added to a constant amount of serum on the amount and composition of the precipitate. The azo-protein used was made by coupling 0·1 g. of amino-euxanthic acid to 0·25 g. of egg albumin. Precipitation was carried out in small centrifuge tubes. Each tube contained 1 ml. of serum, the required volume of azo-protein solution, and 0·9 % sodium chloride solution to bring the total volume to 4 ml. The various solutions were cooled to 0° C, and the mixtures were made in a water-bath containing melting ice. The tubes were left in the refrigerator for 24 hr., when precipitation was complete. The various precipitates were then centrifuged down, the supernatant pipetted off, and the precipitate washed twice in ice-cold 0·9 % saline. The precipitate was then dissolved in a little very dilute sodium hydroxide, transferred quantitatively to a suitable graduated tube, and made up to exactly 1 ml., a trace of caprylic alcohol being added to counteract frothing.

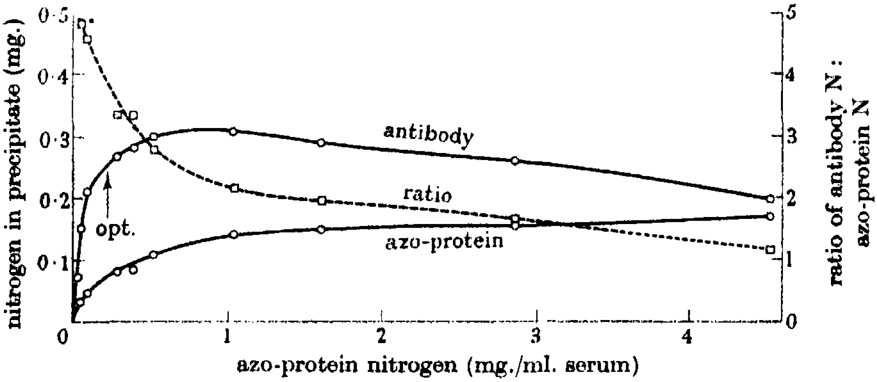


FIGURE 1

The brown solution was then compared in a micro-colorimeter with a suitable dilution, made faintly alkaline, of the original azo-protein solution. From the colorimeter reading and the nitrogen content of the azo-protein solution, the azo-protein nitrogen in the precipitate was calculated. The redissolved precipitate was then washed quantitatively into a hard glass tube, and the total nitrogen determined by the micro-Kjeldahl method of Parnas and Wagner. The difference between total nitrogen and azo-protein nitrogen gave the antibody nitrogen in the precipitate.

The two curves shown in full lines give the antibody and the azo-protein nitrogen in the precipitate. The arrow shows the 'optimum ratio' as determined by the earliest appearance of precipitation. It will be seen that the antibody in the precipitate rises to a maximum and then falls, the highest point being well beyond the optimum ratio. This is what is found with type II antiserum and the polysaccharide, as well as with atoxyl azo-protein and its antiserum (see curves in Woolf, next paper). At very low values of added azo-protein, almost the whole of it is recovered in the precipitate. After that, the amount precipitated rises very slowly. The dotted curve (ordinate values on right) shows the ratio of antibody nitrogen to azo-protein nitrogen in the precipitate. It falls steeply at first, approximately in a straight line. After the maximum of precipitation, the points lie on another straight line, falling much less steeply. Similar curves for the antibody/antigen ratio were obtained by Heidelberger and Kendall (1929) with pneumococcus polysaccharide and its antiserum.

The quantitative relations between the amount of antigen and antibody in the reaction mixture, and the amount and composition of the precipitate, are thus very similar for the specific polysaccharide and the amino-euxanthic acid azo-protein.

Attempts were made to immunize rabbits with amino-euxanthic azo-horse globulin, with a view to testing whether the antiserum would react with the bacterial polysaccharide. The rabbits were immunized by intravenous injections, twice a week, and serum was taken for test after 6 weeks, and thereafter at intervals for several months. None of the three rabbits used gave a serum that would react either with amino-euxanthic acid azo-proteins or with the bacterial polysaccharide. Rabbits vary widely in their susceptibility to immunization with the artificial azo-protein antigens. The failure of these preliminary experiments with only three animals, and with only one azo-protein preparation, does not necessarily mean that amino-euxanthic acid azo-proteins cannot act as antigens. Further work with a larger number of animals and with preparations of azo-protein containing different proportions of amino-euxanthic acid groups is desirable. Experiments were also kindly made by Dr A. W. Downie, in which mice, previously treated with an immunizing course of injections with amino-euxanthic acid azo-protein, were subjected to type II pneumococcus infection, and compared with untreated controls. No protective effect of the azo-protein injections could be observed.

The following experiments show that the serum protein carried down by the azo-protein from type II anti-pneumococcal serum is actually pneumococcus antibody.

A known volume of antiserum was precipitated with azo-protein. The precipitate was centrifuged off, and the antibody nitrogen was determined as described above. The supernatant was then precipitated with the optimal amount of type II specific polysaccharide, and the nitrogen in the precipitate was estimated. The nitrogen precipitated by the polysaccharide from untreated serum was also determined. The results, calculated for 1 ml. of serum, are shown in table 1. It is clear that the azo-protein removes from the serum part of the material precipitable by the specific polysaccharide.

TABLE 1

	mg. N per ml. serum
N precipitated from serum by azo-protein	0·18
N precipitated from supernatant by polysaccharide	0·48
total nitrogen precipitated	0·66
N precipitated from serum by polysaccharide	0·67

The presence of pneumococcus antibody in the azo-protein precipitate was further shown in the following animal tests, very kindly done by Dr A. W. Downie. A known volume of type II pneumococcus antiserum was precipitated with amino-euxanthic acid azo-protein, and the precipitate was centrifuged off, the supernatant being collected. The precipitate was washed, dissolved in sodium euxanthate, and made up to the original volume of the serum with 0·9 % saline. Thus there were three solutions to be compared for pneumococcus antibody—redissolved precipitate, supernatant, and the original serum.

An eight hour culture of a virulent strain of type II pneumococcus was prepared, and injected into mice. One group of mice was injected at the same time with various dilutions of redissolved precipitate, a second group with supernatant, and a third with whole serum. A fourth control group was treated only with the virulent culture. The results are shown in tables 2 and 3. The symbol 0 denotes a mouse that survived for 48 hr., and + a mouse that died during that period.

The number of animals used was too small, of course, for a quantitative assay. But the results show, beyond doubt, that the precipitate from 0·04 ml. of serum contained enough antibody to protect a mouse against half a million times the lethal dose of a virulent culture of type II pneumococcus, and are consistent with the relative protective content of the three solutions suggested by the chemical analysis figures in table 2. There can be no doubt, therefore, that what the azo-protein precipitates from the serum is part of the antibody to type II pneumococcus.

The amino-euxanthic acid azo-protein differs most fundamentally in chemical constitution from the specific polysaccharide in all respects save one, that they have a glucuronic acid grouping in common. Yet the immunological behaviour of the azo-protein towards the serum is very similar, qualitatively and in its quantitative relations, to that of the polysaccharide. The main differences are that the azo-protein precipitates

TABLE 2. CONTROLS

Each mouse was injected with 0·5 ml. of the dilution of the virulent culture shown in column 1.

dilution of culture	
10^{-6}	+ + + + + +
10^{-7}	+ + + + + +

TABLE 3. EXPERIMENTALS

Each mouse was injected with 0·5 ml. of a 1/20 dilution of the culture, and an amount of protective fluid corresponding to the volume of original serum shown in column 1.

protective dose ml.	whole serum	redissolved precipitate	supernatant fluid
0·2	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0
0·04	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0
0·008	0 0 0 0 0	0 0 + + +	0 0 0 0 +
0·001 6	0 0 0 0 +	+ + + + +	0 0 + + +
0·000 32	0 + + + +		

only a part of the antibody, and that polysaccharide precipitation is only partly inhibited by glucuronides, which completely inhibit azo-protein precipitation. The results with amino-euxanthic acid azo-protein therefore afford powerful additional evidence for the belief that glucuronic acid, or some closely related group, is a 'determinant group' responsible for at least part of the specificity between type II pneumococcus antiserum and the type polysaccharide.

It is a pleasure to express my thanks to Dr Panton and the authorities of the London Hospital for permission to work in their laboratories, to the Council of the Royal Society for the Moseley Research Studentship during whose tenure the above work was done, to Dr Downie for kindly doing the animal experiments, and especially to Professor Marrack for his constant daily encouragement, assistance and advice.

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Quantitative studies on the effect of haptenes on the reaction between antigens and antibodies

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Quantitative studies on the inhibition by haptenes of the antigen-antibody precipitate have been made on three systems:

1. Pneumococcus type II polysaccharide and type II antiserum, with sodium euxanthate as inhibitor.
2. Partially hydrolysed cherry gum and type II pneumococcus antiserum, with sodium euxanthate as inhibitor.
3. Atoxyl-azo-egg albumin and atoxyl-azo-globulin antiserum, with atoxyl as inhibitor.

In all three systems the inhibition by a given concentration of haptene varied very little over a wide range of antigen/antibody ratios. While the amount of precipitate depends mainly on the total amounts of antigen and antibody, varying little with dilution, the degree of inhibition depends on the concentration and not the total amount of haptene.

In system 1 there was only partial inhibition even with high concentrations of haptene. The concentration of haptene required to give half the maximum effect was about sixteen times as great as in system 2.

In system 3, the ratio of antibody to azo-protein in the precipitate is lowered by the presence of haptene. There are wide variations in sera from different rabbits in the concentration of haptene required to give the same degree of inhibition.

The bearing of these results is discussed on the nature of the action between antigen and antibody.

Since the discovery by Landsteiner (1920) that the precipitation of a protein containing an artificially introduced 'determinant group' by the specific antiserum is inhibited by small molecules—'simple haptenes'—containing the determinant group, many workers have studied the inhibition phenomenon, and have used it to throw light on a number of aspects of immunology. Thus Landsteiner (1920), Landsteiner and van der Scheer (1936), Goebel, Avery and Babers (1934) and Wormall (1930), among others, have used inhibition to elucidate details of the chemical specificity towards their antisera of antigens containing artificially introduced groups. Marrack and Smith (1932) and Haurowitz and Breinl (1933) have shown by dialysis methods that haptene and antibody actually combine. When, following the isolation of specific polysaccharides from the various serological types of the pneumococcus, cross-precipitation was shown between type II antiserum and certain weakly hydrolysed gums, it was the observation that glucuronic acid or certain of its compounds was a common inhibitor which suggested that glucuronic acid was a 'determinant group' at least partly responsible for the specificity of type II pneumococcus and its antiserum, and led to the work with protein-glucuronic compounds confirming this suggestion (Woolf 1941; Goebel 1936). There are indications, such as the observation of Heidelberger and Kendall (1933), that precipitation of type III pneumococcus polysaccharide by the type antiserum is inhibited by polysaccharide hydrolysis products, that the skilful use of the inhibition technique may afford a great deal more information about the chemical basis of specificity between natural pathogens and their antisera.

Almost all these important results have been obtained by the very rough semi-quantitative technique of judging by inspection, and expressing in terms of plus signs, the amount of precipitate produced under various conditions. It seemed desirable to study the phenomenon on a more strictly quantitative basis. In enzyme chemistry, quantitative studies of the effect of competitive inhibitors have been of the utmost value in elucidating the mechanism of enzyme action. Similar valuable information might reasonably be expected from quantitative studies of immunological inhibition by simple haptenes. The results described in this paper are a first approach into this wide field.

Observations have been made on three systems:

1. Pneumococcus type II polysaccharide and type II antiserum, with sodium euxanthate as the inhibitor.
2. Partially hydrolysed cherry gum and type II pneumococcus antiserum, with sodium euxanthate as inhibitor.
3. Atoxyl-azo-egg albumin and atoxyl-azo-globulin antiserum, with atoxyl as inhibitor.

In systems 1 and 2, the serum used was a potent horse type II pneumococcus antiserum. The type II polysaccharide (hereafter referred to as S.S.S. II) was prepared as described by Felton, Kauffmann and Stahl (1935). The cherry gum was partially hydrolysed by dilute hydrochloric acid at room temperature, as described by Heidelberger, Avery and Goebel (1929).

It has been shown by Marrack and Carpenter (1938) that glucuronic acid and its compounds completely inhibit precipitation by the serum of partially hydrolysed gums, and delay the appearance and reduce the amount of precipitate given with S.S.S. II. The object of the experiments to be described was to obtain a quantitative measure of these effects.

Figure 1 shows the precipitate obtained from 1 ml. of the serum with varying amounts of S.S.S. II. Precipitation was carried out in small centrifuge tubes. Each tube contained 1 ml. of serum, and the required amount of a neutral solution of S.S.S. II in 0·9 % sodium chloride, made up to a volume of 4 ml. with 0·9 % sodium chloride solution. All solutions were cooled in ice, and mixing was done in a water and ice bath. The tubes were left in the refrigerator for 24 hr. The precipitate was then centrifuged down, washed twice in the centrifuge in ice-cold physiological saline, dissolved in dilute alkali, and the nitrogen estimated by the micro-Kjeldahl method of Parnas and Wagner.

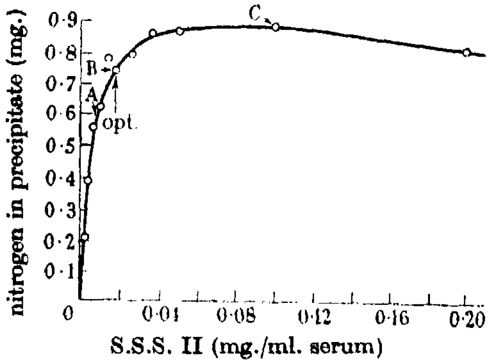

Figure 1

The curve is similar to that given by Heidelberger and Kendall (1929, 1935*a*). The arrow shows the 'optimum ratio' at which precipitation is first observed. The maximum precipitate after long standing is given at a considerably higher S.S.S. II/serum ratio. The maximum amount of nitrogen precipitated from 1 ml. of the serum was 0·88 mg.

Figure 2 shows the inhibiting effect of increasing concentrations of euxanthic acid at 'optimum ratio'. Each tube contained 1 ml. of serum, 0·018 mg. of S.S.S. II (corresponding to point *B* in figure 1) and the required amount of a neutral solution of sodium euxanthate made isotonic with sodium chloride, the volume being brought to 3 ml. with 0·9 % sodium chloride solution. Precipitation and estimation were carried out as described above. The alkaline solution of the precipitate after washing was colourless, showing that it did not retain euxanthic acid.

The curve shows that the maximum inhibition is about 57 %. The concentration of euxanthic acid giving half the maximum effect is about 0·008 M.

Figure 3 shows the inhibiting effect at three different values of the S.S.S. II/serum ratio. Curve *A* was obtained with 0·008 mg. of S.S.S. II per ml. of serum, corresponding to point *A* in figure 1. Curve *B* had 0·018 mg. of S.S.S. II, and curve *C* had 0·1 mg. of the polysaccharide, per ml. of serum, corresponding to points *B* and *C* in figure 1. It will be seen that the inhibiting effect does not vary markedly with widely different amounts of S.S.S. II.

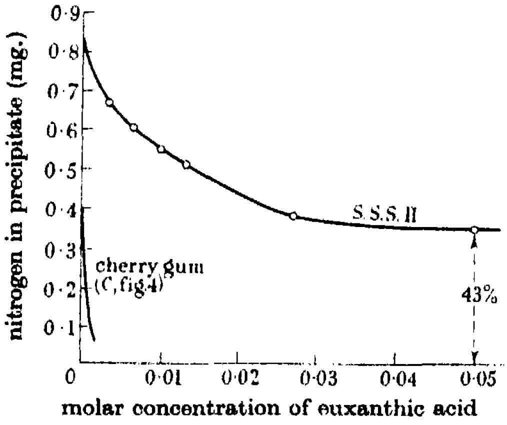

FIGURE 2

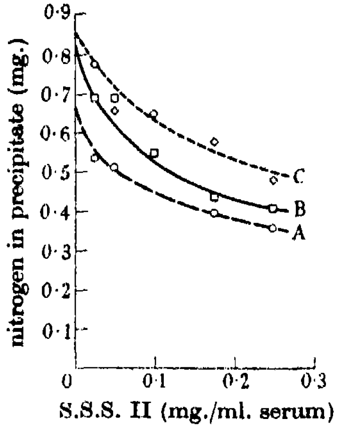

FIGURE 3

Figure 4 shows a similar set of curves, obtained with the weakly hydrolysed cherry gum. The maximum amount of nitrogen precipitated from 1 ml. of the serum by cherry gum was about 0·4 mg., being about 45% of the amount precipitated by S.S.S. II. The 'optimum ratio' was 0·027 mg. cherry gum per ml. of serum. Curve *B* was obtained with the optimum ratio, curve *A* with half the quantity of gum (0·0135 mg. per ml. of serum) and curve *C* with excess of gum (0·1 mg. per ml. of serum). The total volume in each tube was 4 ml.

The figure shows that the inhibiting effect is not greatly altered over a wide range of gum/serum ratios. Curve *C* is somewhat steeper than the others, but the effect is not very big, as shown by the following values read off from the curves:

TABLE 1

mg. cherry gum per ml. serum		molar concentration of euxanthate giving 50% inhibition
A	0·0135	0·000 52
B	0·027	0·000 55
C	0·1	0·000 42

The figure also shows that the cherry gum precipitation is much more easily inhibited than that with S.S.S. II, which required about sixteen times as much euxanthate to give half the maximum effect. Curve *C* of figure 4 is shown in figure 2, so that the relative effects can be compared.

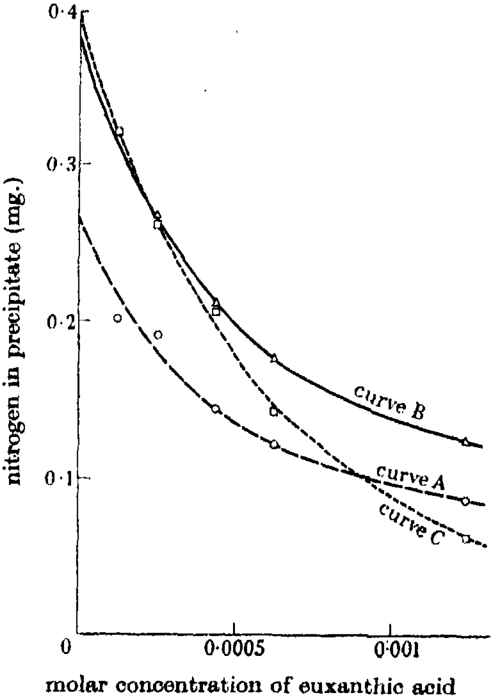

FIGURE 4

Both with the S.S.S. II and with the gum (Marrack and Carpenter 1938), the amount of precipitate depends on the quantities of serum and polysaccharide, and is very little affected by dilution of the reaction mixture. In order to test whether the inhibiting effect of the haptene depends on the concentration or the total amount in the mixture, the following experiment was done. Six tubes were put up, each containing 1 ml. of serum and 0·027 mg. of cherry gum. Tubes 2, 4 and 6 contained in addition 0·5 ml. of 0·004 M euxanthate. The total volumes were made up with saline as shown in table 2.

The amount of precipitate in the uninhabited tubes (nos. 1, 3, 5) is seen to be independent of concentration within the range of 2–8 ml. The degree of inhibition, on the other hand, varies with haptene concentration. The results are plotted in figure 5. The curve is curve *B* of figure 4, showing the inhibiting effect of euxanthate in the same gum-serum mixture in a constant volume of 4 ml. The points fit well on the curve, showing that the inhibiting effect of the haptene depends only on the concentration, and not on the total amount in the mixture.

TABLE 2

tube no	total volume ml.	euxanthate concentration M	nitrogen in precipitate mg.
1	2	—	0·39
2	2	0·001	0·15
3	4	—	0·38
4	4	0·000 5	0·19
5	8	—	0·39
6	8	0·000 25	0·27

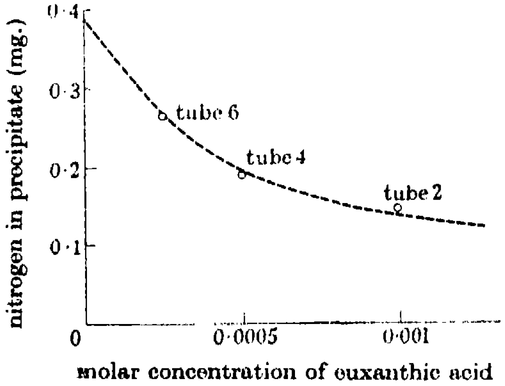

FIGURE 5

In the third system studied, the antiserum was obtained from rabbits immunized with horse globulin coupled with diazotized atoxyl (*p*-aminobenzene-arsinic acid). The test antigen was atoxyl-azo-egg albumin. The inhibitor was atoxyl. This system has the advantage that both antigen and antibody can be estimated in the precipitate, so that the effect of the haptene can be measured on the composition as well as the amount of the precipitate. It has, so far as these experiments are concerned, the disadvantage that serum prepared from a large animal like the horse was not available. Only a limited number of tests can be carried out on the serum obtained from one bleeding of a rabbit; and the properties of the sera obtained from successive bleedings of a single rabbit are not identical, still less the sera from different rabbits.

The azo-protein for immunization was made by coupling 0·1 g. of atoxyl to 1 g. of horse serum globulin. For the test antigen, 0·4 g. of atoxyl was coupled to 1 g. of twice recrystallized egg albumin. After coupling was complete, the azo-protein was purified by being several times precipitated with dilute hydrochloric acid and redissolved in dilute sodium hydroxide. followed by dialysis in the refrigerator against several changes of 0·9 % sodium chloride solution. Any precipitate formed was centrifuged off. The azo-protein solution so obtained was a neutral, clear red fluid, isotonic with serum.

The rabbits were immunized by intravenous injections of the azo-globulin solution, twice a week for six weeks.

The haptene solution consisted of atoxyl neutralized with sodium hydroxide, containing enough sodium chloride to make it isotonic with serum.

Precipitation was carried out in centrifuge tubes as described above. For estimation, the precipitate after washing was dissolved in a little dilute sodium hydroxide, transferred quantitatively to a measuring vessel, and made up with water to 1 ml. It was then compared in a micro-colorimeter with a suitable dilution of stock azo-protein solution, made alkaline with sodium hydroxide. This procedure was tested by precipitating the azo-protein with a large excess of serum, when the antigen is all precipitated. The colorimeter readings accorded closely with the amount of azo-protein in the mixture.

The azo-protein nitrogen in the precipitate was calculated from the colorimeter reading and the nitrogen content of the test antigen solution. The whole of the precipitate was then washed into a hard glass tube, and the total nitrogen estimated by the micro-Kjeldahl method of Parnas and Wagner. The difference between the two figures is the antibody nitrogen. The term 'A/P ratio' will be used to denote the ratio of antibody nitrogen to azo-protein nitrogen in the precipitate.

Table 3 gives some results with a serum (rabbit 622) whose 'optimum ratio' was about 0·085 mg. of azo-protein nitrogen to 1 ml. of serum. Each tube contained 1 ml. of serum and the stated amount of azo-protein and haptene, in a total volume of 4 ml.

It will be seen that the A/P ratio falls in presence of haptene at least with the lower concentrations of antigen. This result may seem paradoxical. It might be expected that the haptene, by competing with the azo-protein for, and blocking some of, the receptive areas on the antibody, would cause each unit of antibody to take up less azo-protein, so that the ratio would rise. This question is further discussed below.

TABLE 3

azo-protein nitrogen mg.	molar concentration of atoxyl	antibody nitrogen mg.	inhibition %	A/P ratio in precipitate
0·036	—	0·425	—	12
0·036	0·000 125	0·149	65	7½
0·036	0·000 25	0·093	78	7
0·096	—	0·474	—	9½
0·096	0·000 25	0·134	72	6
0·096	0·000 5	0·086	82	6½
0·120	—	0·483	—	9
0·120	0·000 25	0·167	56	9

Results obtained with another batch of serum from rabbit 622 are shown in figures 6 and 7. Figure 6 shows the effect of varying amounts of azo-protein on the quantity and composition of the precipitate obtained with 1 ml. of serum, the total volume in each tube being 4 ml. The full lines (scale on left) show the antibody nitrogen and the azo-protein nitrogen in the precipitate. The broken line (scale on right) shows the A/P ratio.

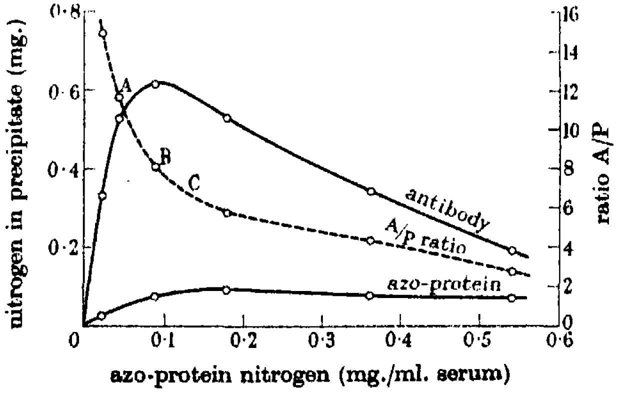

FIGURE 6

It will be seen that this ratio falls steeply at first, and then, after the maximum precipitation point, the graph forms a much less steep straight line. Similar curves for the A/P ratio were obtained by Heidelberger and Kendal (1935*b*) for an azo-protein and rabbit serum, and by Woolf (1941) for amino-euxanthic acid-azo-protein and type II pneumococcus antiserum.

Figure 7 shows the inhibiting effect of atoxyl on a mixture corresponding to point *A* on figure 6. The numbers on the curve give the A/P ratio at each point. This ratio falls at first, but then rises slightly.

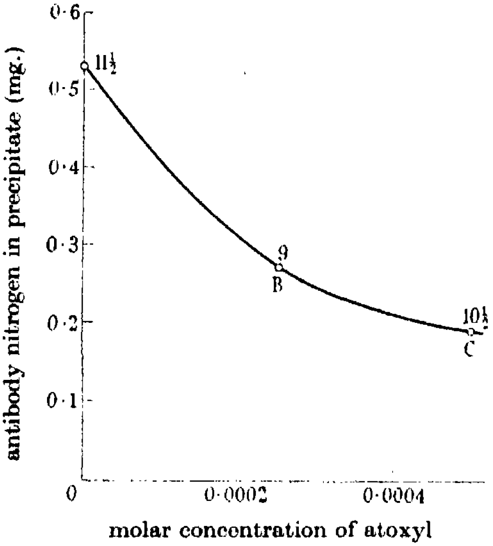

FIGURE 7

The following experiment was done to test the reversibility of the union between antigen and antibody in presence of haptene. Three tubes were put up, each containing 1 ml. serum and azo-protein of nitrogen value 0·045 mg., the mixture used for the curve in figure 7. The tubes were treated as shown in table 4:

TABLE 4

	initial composition			final composition	
tube no.	volume ml.	atoxyl concentration	added after 24 hr.	volume ml.	atoxyl concentration
1	4	—	—	4	—
2	4	0·000 25 M	—	4	0·000 25 M
3	3	—	1 ml. of 0·001 M atoxyl	4	0·000 25 M

The tubes were kept in the refrigerator for 3 days. After the addition to tube 3, the precipitate was rubbed up several times a day with a glass rod. The usual estimations were made, giving the results shown in table 5:

TABLE 5

tube no.	antibody nitrogen, mg.	inhibition %	A/P ratio
1	0·53	—	11½
2	0·29	49	9½
3	0·34	36	8½

Addition of the haptene after the precipitate is fully formed gave three-quarters of the inhibiting effect found in tube 2, and the characteristic fall in the A/P ratio. This accords with the observation that the precipitate is completely soluble in high concentrations of haptene. It is possible that complete reversibility is prevented by the difficulty of rubbing up the precipitate so that the haptene has full access to all of it.

Figure 8 shows the difference in the inhibiting effect of atoxyl, under comparable conditions, on sera from different rabbits. The curve marked 'serum 622' is that shown in figure 7. The other curve was obtained in a similar way with serum from rabbit 428. The numbers give the A/P ratios. It will be seen that serum 428 is much less easily inhibited. The atoxyl concentrations giving 50 % inhibition are as follows:

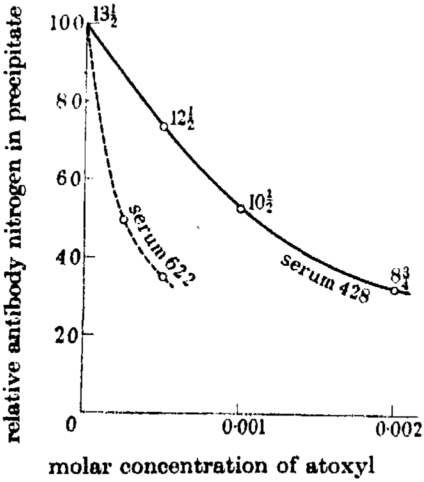

FIGURE 8

serum 622	0·000 25 M
serum 428	0·001 15 M

There is thus nearly a five-fold difference.

DISCUSSION

The results described above demonstrate the validity of quantitative methods of studying inhibition, and open out a number of new lines of inquiry.

In comparing the behaviour of the specific polysaccharide and the gums towards the pneumococcus antiserum, the following differences are observed:

The polysaccharide gives more precipitate than the gums.

The polysaccharide precipitate appears much more quickly than that with the gums. It is also probably true that the gums stand in the same order among themselves for minimum time of first formation of precipitate and for total amount of precipitate finally formed (Marrack and Carpenter 1938).

The polysaccharide requires far more haptene than the gums for the same relative degree of inhibition. The gums stand in the same order among themselves for amount of precipitate in absence of inhibitor and for concentration of haptene to give the same degree of inhibition (Marrack and Carpenter 1938).

The polysaccharide precipitate is only partially, and the gum precipitates wholly, inhibited by excess of haptene.

The first three of these differences suggest the possibility, to be tested by further experiments, that speed of precipitate formation, total amount of precipitate, and ability to resist inhibition, are three aspects of the same phenomenon, the relative avidity of the antibody for the antigen.

It is not to be expected that haptene inhibition of an immunity reaction should show the same quantitative features as competitive inhibition of an enzyme action. The action between antigen and antibody, depending on amount of each present and not on concentration, giving a stable insoluble antigen-antibody complex, and showing the zone phenomenon, is vastly different to the action between enzyme and substrate, which depends on concentration, gives a soluble unstable enzyme-substrate intermediate compound, and, at any rate in many cases, is not inhibited by excess of substrate.

It may be assumed, however, on the basis of the dialysis experiments, that the haptene competes by forming a reversible compound with the receptive areas of the antibody. If the first stage in precipitate formation is a reversible combination between antigen and antibody, the impeding effect of a given concentration of haptene will vary inversely with the rate of this combination. The haptene may, of course, also interfere with the later stages that give rise to the insoluble precipitate. If this is the case, then velocity of precipitate formation and resistance to inhibition should, for a series of antigens with the same serum, always vary together. The results so far found by previous workers, and those described above, are consistent with this view. But it needs to be more thoroughly tested.

If such a view is valid, it gives the quantitative measure of inhibition an importance as being a measure of the affinity of antibody for antigen. The total amount of precipitate with cherry gum, in the experiments described above, was nearly half that with the specific polysaccharide. But the concentrations of haptene needed to give 50 % of the maximum inhibition varies as 16 : 1. Inhibition is thus a far more sensitive index than total precipitate. Time of first appearance of precipitate is also a more sensitive indicator than total precipitate, but it is difficult to measure quantitatively.

The fall in the A/P ratio with increase of haptene calls for some discussion. It might be expected that the haptene would increase the ratio, as explained above. But the following hypothesis, which is capable of experimental test, may be tentatively put forward. The haptene causes the total amount of precipitate to decrease. Its action may therefore, to a first approximation, be divided into two effects:

1. Removing a proportion of antibody entirely from the sphere of action, by blocking it up and rendering it inaccessible to antigen.

2. In that portion of the antibody which does combine with antigen, blocking some of the receptive areas and so decreasing the number of antigen particles taken up per unit of antibody.

The first of these effects would cause the A/P ratio to fall (see dotted curve in figure 6). The second would cause it to rise.

If this explanation be true, the fall in the A/P ratio should be less than that attributable to the first effect alone. The data, so far as they go, are partly consistent with this view. Thus, in figure 7, the A/P ratios at points *B* and *C* are 9 and 10½. If the inhibition were caused only by the removal of a proportion of antibody from the sphere of action, the effective mixtures would correspond to points *B* and *C* in figure 6, and the ratios would be 8 and 7. On the other hand, some of the results shown in table 3 do not appear to be consistent with this hypothesis. It is obviously necessary to investigate the question more thoroughly. It is difficult to do so with rabbit serum, where only a few estimations can be done with the product of one bleeding. A suitable system would be pneumococcus type II antiserum and amino-euxanthic acid-azo-protein, with euxanthic acid as inhibitor.

The fact that partial inhibition only is obtainable with the specific polysaccharide may be explained in two ways. It is possible that there is some other determinant group in the molecule besides glucuronic acid, responsible for part of the specificity. The fact that precipitates are obtained from the antiserum with flax-seed mucilage, which are not inhibited by glucuronides (Marrack and Carpenter 1938) would support this view. The other possibility is that the determinant group is closely related to, but not identical in configuration with, the glucuronic acid residue as present in euxanthic acid, and that only those antigenic combining groups of a somewhat wide specificity are inhibited. This view is consistent with the findings of Landsteiner and van der Scheer (1936). It may be possible to decide between these alternatives in the following ways. First, by attempting to find the hypothetical determinant group common to flax-seed mucilage and the specific polysaccharide, by testing for inhibiting power other chemical groupings present in the polysaccharide molecule. Second, it should be possible, by precipitating the serum with small quantities of the polysaccharide in presence of excess haptene, centrifuging off the precipitate and dialysing off the haptene, to separate the 'non-glucuronic-sensitive' portion of the serum. This could then be tested to see whether it differed in specificity and sensitiveness to inhibitors from the whole serum.

The above are some of the lines of work opened out by the preliminary studies described in this paper. It seems reasonably certain that more extended investigations of the quantitative effects of haptenes under different conditions will yield results throwing a great deal of light on the mechanism of immunity reactions.

It is a pleasure to express my thanks to Dr Panton and the authorities of the London Hospital for permission to work in their laboratories, to the Council of the Royal Society for the Moseley Research Studentship during whose tenure the above work was done, and especially to Professor Marrack for his constant daily encouragement, assistance and advice.

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The electrical polarization of frog skin by direct currents

By O. Gatty*

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Results concerning the electrical polarization of frog skin by direct currents are presented with adequate statistical control. The results are discussed and show that the skin is electrically non-homogeneous and in a state of disequilibrium. The results are presumptive evidence for at least a three-unit model consisting of a series resistance and local action between an anodic area and a cathodic area as is also considered in the theory of corroding metals.

Introduction

The properties of interphases and of membranes can be studied by observing their behaviour when subjected to electrical polarization. The use of direct currents over short periods of time gives information from a single experiment comparable to a series of observations with alternating current of varying frequency, and over longer periods allows observations to be made of the effect of the concentration changes produced by electrolysis. Investigations of solution-metal interphases have been undertaken on these lines by many workers (for references see Gatty and Spooner 1938), both under conditions of constant polarizing potential and of constant polarizing current. The latter method has the advantage that the current is constant across every membrane and electrode in the electrolytic system, so that measurements across individual membranes refer to time variations of potential under simpler conditions since the potentials are not constant, in the general case, across all membranes when the former method is used; an additional simplification is that the coulombs that have passed vary directly with the time. For tissues that do not exist in the form of a homogeneous membrane the advantages of using constant current are not so great since the distribution of current between different parts of a mosaic membrane may vary with the time, although the total current density across the membrane as a whole is kept constant.

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* *Note by communicator.*—This communication by O. Gatty was virtually completed before his death, due to an accident in work which he was carrying out on behalf of his country. In revision and preparing for Press I have taken the liberty of making a few textual alterations.

Frog skin has a complex structure as revealed under the microscope, and evidence that it is electrically non-homogeneous is presented in this paper; it follows that problems of topochemistry are of considerable importance in the interpretation of polarization data. A satisfactory interpretation of the electrical behaviour of frog skin becomes, therefore, a matter of extreme complexity, and the establishment of definite conclusions needs the collection of a quantity of experimental material.

The experimental methods and results are given below, and it is shown that these results indicate that the skin is electrically non-homogeneous. An outline of how the results might be interpreted in terms of local action currents—an idea familiar to all workers on metallic corrosion—is then given to illustrate the lines on which more detailed interpretation is likely to evolve.

EXPERIMENTAL

The methods of preparing the frog skin and of making the Ringer solutions were similar to those of Francis and Gatty (1938). The skins were mounted in cells of the type shown in figure 1. The skin *SS* is mounted between two glass half-cells, the polarizing current flows between the electrodes *P* and *P'*, contact with the solution being made through mercury cups and platinum wires sealed into the glass, and the measuring electrodes were saturated calomel electrodes dipping into two beakers of frog's Ringer solution into which also dipped respectively the ends *A* and *A'* of the half-cells. Renewals and changes of solution were made from the vessels *B* and *B'* into the cell and out through the outlets *O* and *O'* to which clipped rubber tubes were attached to prevent flow of solution from the beakers through *A* or *A'* into the half cells. Fresh solution was run through both compartments immediately before and directly after each polarization;

the solution in contact with the inner side was renewed first in each case. Measurements of potential were taken 15 sec. after applying a polarizing current and 15 sec. after cutting it off. All other measurements of potential refer to successive half-minutes before or after some change in the polarizing current density. In the curves the zero of time is the instant of switching on of the polarizing current.

Constant polarizing currents were obtained by rapidly closing a mercury contact switch which was in series in the polarizing circuit with 2 megohms, the cell, a micro-ammeter, and a high-tension battery. It was estimated that the inductance of the polarizing circuit would allow a constant current to be set up after less than 10^{-12} sec., so that the rapidity of closing the switch may well have been the limiting factor in the rate of setting up a constant current. Since the resistance of a complete cell mounted with skin is only some 2000 ohms, fluctuations in the total resistance of the circuit due to changing properties of the skin should be small. For the majority of measurements the calomel electrodes were connected through a potentiometer to a null instrument which was a megohm in series with a string galvanometer.

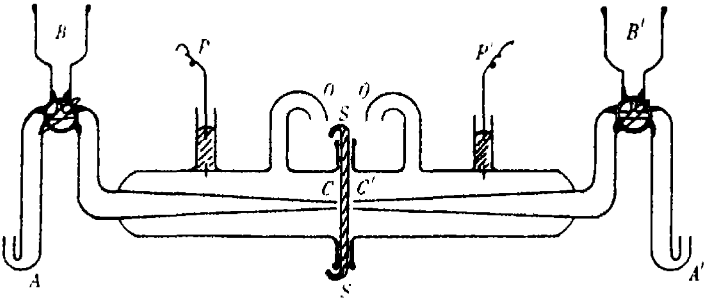

FIGURE 1

Measurements of skin resistance and conductance are corrected for the resistance of the solution between C and C', which varied from 129 to 53·5 ohms according to the cell, and are expressed respectively in ohms-cm.$^{+2}$ of cross-section of the cell and reciprocal megohms-cm.$^{-2}$ of cross-section of the cell. In some cases where the skin was not stretched during mounting, the surface area of the skin may have been up to 20 % more than the cross-section of the cell which was about 0·8 cm.^{2}. The potential fall across the skin is taken as the potential of the calomel electrode in the solution in contact with the inner side of the skin less that of the electrode in the solution in contact with the outer side of the skin. Polarizing currents will be said to be positive or anodic when positive current flows externally into the solution in contact with the inner side of the skin, through the skin, and out through the solution in contact with its outer side.

Statistical control

Frog skin, as prepared for the present work, is a variable material since its potential and its conductance cm.$^{-2}$ have standard errors per observation, of some 30 %, while certain features of the polarization curves show still greater variability—possibly because they depend on the effects of several factors each of which has a variance of the order of 30 %. The present work confines itself to observations that are significant to the 5 % level of significance or better from ten comparisons (or ten experiments) or from a smaller number of experiments. The statistical tests employed were those of Fisher's analysis of variance and of covariance (Fisher 1936).

An idea of the method is obtained by considering the most usual components into which the variance was analysed. Observations were made (*a*) on the skin of more than one *frog*, (*b*) on each skin on more than one *occasion*, and (*c*) on two *control* skins symmetrically taken from the belly of a single frog. The control pairs were sometimes subjected to identical treatment but more usually to differing treatment. The variance was then analysed into (1) variance between frogs, (2) variance between occasions, (3) variance between controls, (4) variance due to frog-occasion interaction, (5) variance due to frog-control interaction, (6) variance due to occasion-control interaction, and (7) variance due to frog-occasion-control interaction. The latter was generally taken as an estimate of error. When the two controls were treated differently, however, results are only mentioned when the variance between treatments (i.e. between controls) per degree of freedom exceeded significantly the variance per degree of freedom in any and all of the other six categories.

The shape of the polarization curves

From August to December 1937 the skins of several frogs were polarized, and time-potential curves are shown in figures 2 and 3. In figure 2, curve *A* is the mean of eight observations at a constant current density that varied between individual cases from $-12\cdot2$ to $-15\cdot2\,\mu$A-cm.$^{-2}$ of tube cross-section. In figure 2, curve *B* is the mean of seventy-six observations at current densities between -25 and $-31\,\mu$A-cm.$^{-2}$; curve *C* is the mean of fifty-one observations at current densities between -53 and $-59\,\mu$A-cm.$^{-2}$; and curve *D* is the mean of fifteen observations at current densities between -82 and $-88\,\mu$A-cm.$^{-2}$. In figure 3, curve *A* is the mean of fifteen

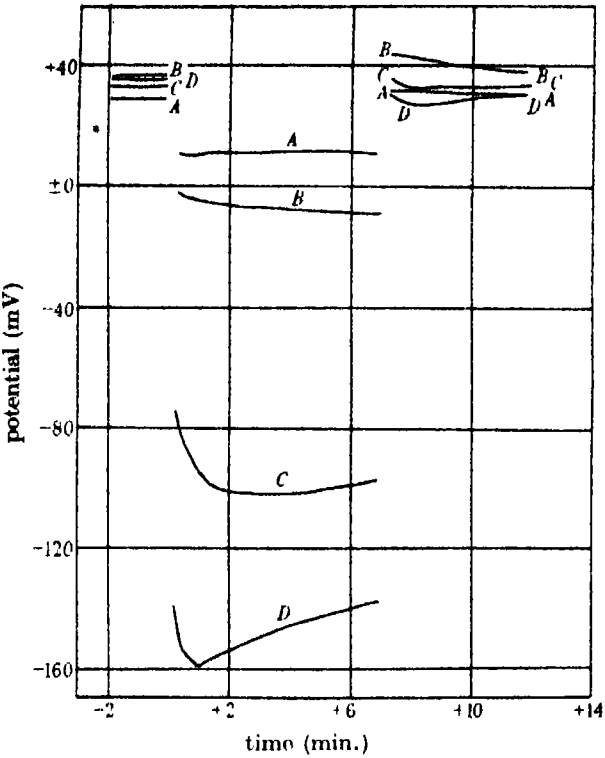

FIGURE 2

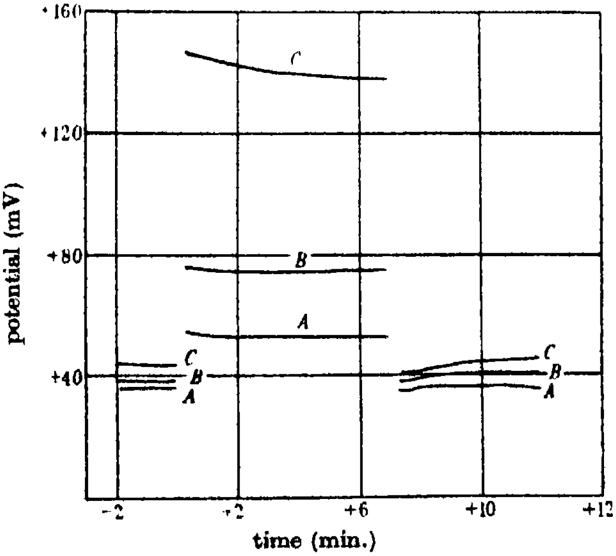

FIGURE 3

observations at current densities between 12·2 and 15·2 μA-cm.$^{-2}$; curve *B* the mean of 129 observations at current densities between 25 and 31 μA-cm.$^{-2}$; and curve *C* is the mean of fifteen observations at current densities between 53 and 59 μA-cm.$^{-2}$.

Features of the curve before polarization

The potential fall is distributed in an approximately normal distribution as judged by the third and fourth moment tests around a mean of + 38 mV. The skin is variable as regards potential fall and the use of controls off the same frog in general leads only to small increases in the accuracy of an experiment (provided that the frog is reasonably healthy). Rough treatment of the skin while mounting in the cell was tested once or twice and showed little effect on the potential fall; thus the variability between controls seem to be inherent in the skin and not to be an artefact. Seasonal and sexual differences were not detected over the period in question.

The potential rises from time − 2 min. to 0. This is presumably due to the renewal of the Ringer solution in the cell at times between − 5 and − 3 min. The mean effect is a rise of 0·4 mV significant to the 1 % level, but falls of potential are frequently observed with individual frogs and a fall of 0·1 mV is observed with the mean of fifteen observations in the case of curve *C*, figure 3.

The change in potential after 15 *sec. polarization*

The change in potential due to the polarizing current was first measured after the current had been on for 15 sec. except in later experiments with a cathode-ray oscillograph. The former observations, when combined with Ohm's law, give a measure of the electrical resistance of the cell between planes perpendicular to the axis of the tubes of the half-cells and passing respectively through *C* and *C'* (figure 1). From these figures it was found that the mean conductance of the skin cm.$^{-2}$ was 817 reciprocal megohms, the mean being taken over many frogs and over successive occasions for each frog separated by 40 min. intervals from 20 to some 220 min. after mounting the skins in the cells.

The conductance decreases with the time after mounting by some 216 reciprocal megohms per hour. The rate of decrease eventually must become less as the skins get older since negative conductances are not possible, and in very old and dead skins the conductance rises again to values higher even than those observed with freshly mounted living skins. The mean polarization curves for five frogs killed in December 1936 are shown in figure 4. The polarization was at current densities between − 25 and

$-31\,\mu$A-cm.$^{-2}$ and was maintained for 12 min. Successive polarizations were done at intervals of 1 hr. and the mean curves for the 1st, 3rd, 5th and 7th polarizations respectively are plotted as curves 1, 3, 5 and 7 in figure 4.

No significant interclass correlation was found between the potential at time 0 and the skin conductance estimated from potential readings 15 sec. later; it seems therefore that these two quantities vary independently.

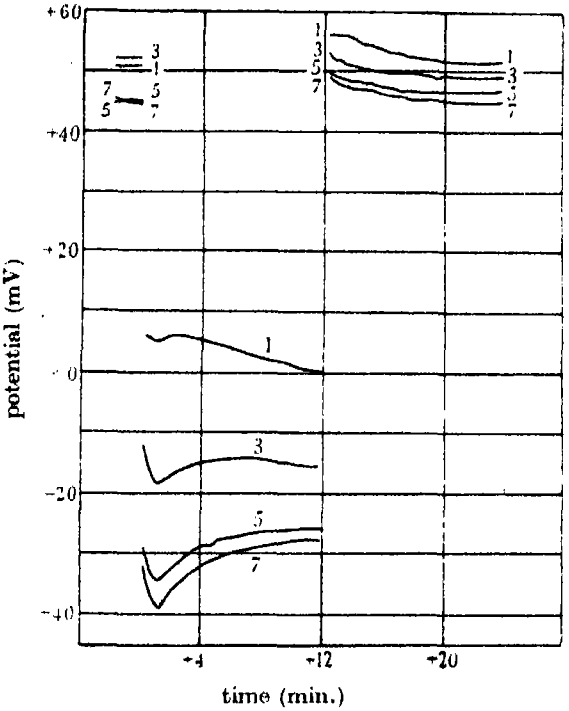

FIGURE 4

The change in potential as a result of polarization

Let the potential at time t be denoted by E_t, and if at time t zero polarizing current is flowing let it be denoted by E^0_t. Curve A, figure 5, gives the values of $E^0_{AP}-E^0_{BP}$ ($E^0_{\text{after polarization}}-E^0_{\text{before polarization}}$) plotted against current density. The sign of $E^0_{AP}-E^0_{BP}$ as shown by the curve is significant in every case. At current densities of either sign but between 25 and $31\,\mu$A-cm.$^{-2}$ in magnitude the duration of the polarizing current has no significant effect on this quantity when tested for durations from 2 to 12 min. At all current densities $E^0_{AP}-E^0_{BP}$ becomes more negative with time after mounting the skin, while at current densities between $+25$ and

$+31\,\mu$A-cm.$^{-2}$ the variance between the frogs for $E^0_{AP} - E^0_{BP}$ decreases with the time.

Accompanying the change in potential on polarization is a change in conductance. At current densities -25 to $-31\,\mu$A-cm.$^{-2}$ the analysis of covariance shows that $(E^0_{AP} - E^0_{BP})$ and $(\Lambda_{AP} - \Lambda_{BP})$ have a significant negative covariance of their mean to the 1 % level when the test is made for variations (*a*) between frogs, (*b*) between occasions, (*c*) in the residual variance which corresponds to frog-occasion interaction where the conductances cm.$^{-2}$ before and after polarizations are denoted respectively by Λ_{BP} and Λ_{AP}.

The change in conductance as a result of polarization

At current densities from -25 to $-31\,\mu$A-cm.$^{-2}$, $(\Lambda_{AP} - \Lambda_{BP})$ is negative; in addition, its magnitude decreases with the time after mounting of the skins, and this result is consistent with the correlations and other results of the last section. At current densities from $+53$ to $+59\,\mu$A-cm.$^{-2}$, $(\Lambda_{AP} - \Lambda_{BP})$ is positive.

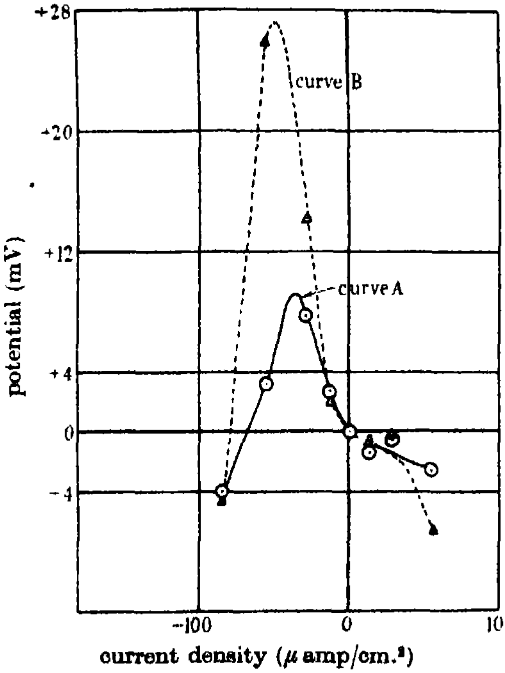

FIGURE 5

The negative correlation of $(\Lambda_{AP} - \Lambda_{BP})$ with $(E^0_{AP} - E^0_{BP})$ has already been mentioned. It is further emphasized by plotting $(E^0_{7\frac{1}{4}} - E_7) - (E^0_0 - E_{\frac{1}{4}})$, which should measure approximately a quantity proportional to the increase of skin resistance during 7 min. polarization, against current density on the same graph as $E^0_{7\frac{1}{4}} - E^0_0$ is plotted against current density. The former curve should show a positive correlation with the latter and becomes curve *B* of figure 5.

Changes occurring in the potential after the flow of polarizing current has ceased

At all the current densities shown in figure 3, $E^0_{7\frac{1}{4}} < \mathrm{E}^0_0$ and also $E^0_{9\frac{1}{4}} > E^0_0$. This suggests that two factors affect the potential as a result of anodic polarization; the first factor reduces the potential and predominates directly after removing the polarizing current. The inequalities stated are read directly from the curves and might not be significant if subjected to statistical analysis, particularly as a relatively large standard error is to be expected if the inequalities are the results of two opposing effects of comparable magnitude and whose time of appearance is subject to variation. Curve *D* in figure 2 also shows the effect of two opposing factors operating on the potential as a result of cathodic polarizations at -82 to $-88\,\mu$A-cm.$^{-2}$.

Changes of potential during polarization

Changes of potential during polarization are most marked during cathodic polarization and also show evidence of two opposing factors. The potential tends to fall at first and subsequently to rise especially at high current densities, but a more complicated variant is shown in figure 4, curve 1. Individual skins show other variations, and the potential may rise steadily from time $\frac{1}{4}$ to time 7 at a current density of -25 to $-31\,\mu$A-cm.$^{-2}$.

Polarization curves that are interrupted periodically to ascertain E^0 values indicate that the initial fall in E is accompanied by a rise in E^0 and in fact corresponds to an increased resistance. The subsequent rise of E has not been investigated in this way, but E^0_{AP} is known to be independent of the duration of polarization between 2 and 12 min. to a low order of accuracy so that the subsequent rise in E probably corresponds to a fall in resistance. The effect of duration of polarization on E^0_{AP} needs to be investigated at high cathodic current densities.

Anodic polarization leads to a fall in E at 53 to $59\,\mu$A-cm.$^{-2}$ (curve *C*, figure 3) but has little effect on mean curves at lower current densities. Some sets of skins show falling and some rising values for E at 25 to $31\,\mu$A-cm.$^{-2}$.

Skin conductance and current density

It is found that the change in potential $(E^0_0 - E_t)$ leads to skin conductances that are independent of the current density over the range $+56$ to $-28\,\mu$A-cm.$^{-2}$. At the two higher cathodic current densities tried the conductances were found to be lower, but an inspection of curves C and D in figure 2 shows that the value of E is changing so rapidly with the time that it is possible that $(E^0_0 - E_{2\text{ sec. or less}})$ would lead to a conductance that was independent of the current density. These observations are to be compared with those of Pumphrey (1934).

Short-period polarizations

A few short-period polarization curves were obtained, with the collaboration of Hubbard and Lord Rothschild, by means of a battery-coupled amplifier and cathode-ray oscillograph made by Hubbard, Rawdon-Smith, and Lord Rothschild. They give definite evidence of two time constants, the one of a period less than 1 msec. and the other with a period of some 15 msec.

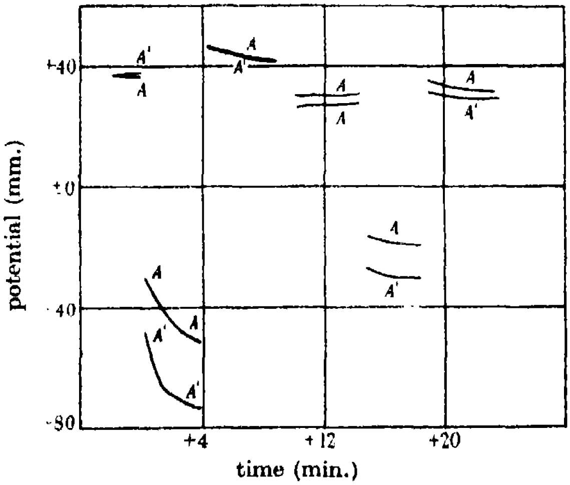

FIGURE 6

The pressure effect

An excess of hydrostatic pressure of 6 cm. of Ringer solution on the inner side of the skin (positive pressure gradient) gives a higher potential than the potential for the same skin with the pressure gradient reversed. The fall of potential on applying a more negative pressure gradient gets reduced with time after mounting the skin. A positive pressure gradient decreases the skin conductance. The effect of a pressure gradient on skin conductance persists in dead and rotting skins whose potential is zero at all pressure gradients.

Positive pressure gradients increase the rise in $(E^0_{AP} - E^0_{BP})$ on cathodic polarization and decrease the negative quantity $(\Lambda_{AP} - \Lambda_{BP})$ after cathodic polarization. This is another example of the negative covariance of $(E^0_{AP} - E^0_{BP})$ and $(\Lambda_{AP} - \Lambda_{BP})$; this time it is observed for variations of pressure gradient across the skin.

It has been known for some time that a pressure gradient across frog skin affected the potential (Motokawa 1933; Gatty and Rawdon-Smith 1937). The skins of four frogs were subjected to cathodic polarization at current densities between -25 and $-31\,\mu$A-cm.$^{+2}$. From the time -2 to 9 min. there was a positive pressure gradient which was then reversed before completing the curves from time 10 to 23·5. The mean curve of these four skins is curve A in figure 6; curve A' is a repetition of curve A after an interval of 64 min.

Tap water and Ringer frogs

It seemed possible that the change of the medium in contact with the outer side of the frog skin from Cambridge tap water (living frogs were kept in this) to Ringer at pH 8 might be responsible for the age effect on the conductance. Thus a test was made of the age effect on conductance on frogs that had been kept for a week in Ringer solution that was changed once a day. In figure 7, curves A, A' and A'' refer to the mean of five frogs on successive occasions separated by intervals of 40 min. These frogs were kept in tap water prior to killing and had their water changed every day for a week beforehand (the skins in both cases were mounted with Ringer at pH 8 on both sides). In figure 7, the curves B, B' and B'' refer to a similar experiment performed at the same time but on the skins of five different frogs which had been kept in Ringer solution for a week prior to killing. The frogs kept in tap water show the same general features in their polarization curves in Ringer solution at -25 to $-31\,\mu$A-cm.$^{-2}$ as other skins, although the polarization was only maintained for 2 min. Thus $E^0_{2\frac{1}{2}} > E^0_0$. Similarly $\Lambda_2 < \Lambda_0$. Also both Λ_2 and Λ_0 decrease with age. The decrease of Λ_2 and Λ_0 with age is obtained also with the Ringer frogs so that the age effect on resistance is not confined to tap-water frogs. For the Ringer frogs, however, $E^0_{2\frac{1}{2}}$ is not significantly greater than E^0_0, and Λ_2, though significantly less than Λ_0, is smaller than in the case for tap-water frogs. This effect is also shown by comparing $E_{\frac{1}{2}} - E_2$ in the case of the two types of frog. In this case too it is evident that there is a correlation between the effect of Ringer in reducing the positive $E^0_{2\frac{1}{2}} - E^0_0$ and increasing the negative $A_8 - A_0$.

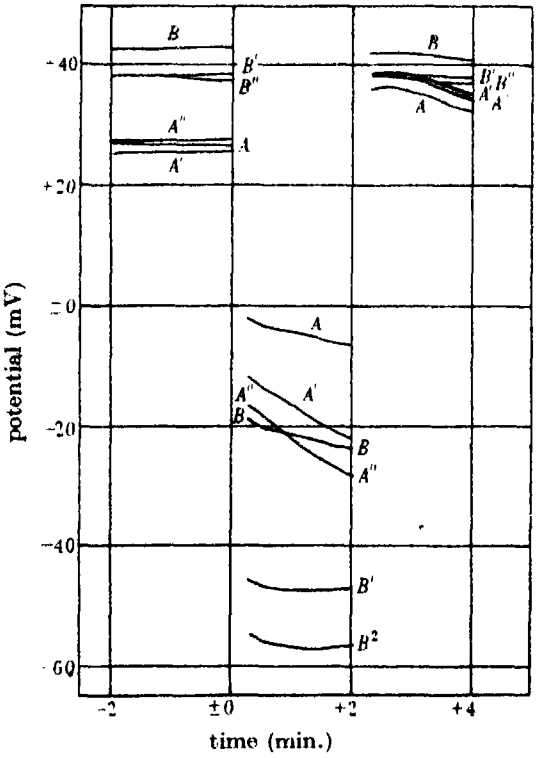

FIGURE 7

Dilute solutions where the outer side solution is M/500 KCl

Provisional work has been done on polarizing frog skin with the Ringer solution at pH 8 in contact with the inner side of the skin but the external solution M/500 KCl. As previously reported (Dean and Gatty 1937; Dean 1939) these conditions produce negative skin potentials. In figure 8, curves *A* and *C* represent the mean curves for polarizing four tap-water frogs at current densities respectively of plus and minus 25–31 μA-cm.$^{-2}$. Curves *B* and *D* represent the mean of three Ringer frogs (kept in Ringer for 7 days before killing) polarized under comparable conditions. $E^0_{7\frac{1}{2}}$ for curve *A* is not significantly positive, but it is greater than $E^0_{7\frac{1}{2}}$ for curve *B* and for curve *A* $E^0_{7\frac{1}{2}} > E^0_0$. For curve *B*, $E^0_{7\frac{1}{2}} > E^0_0$ (on curve, but it has not been found significant with these few observations).

The magnitude in the changes in E during anodic polarization suggests that E^0 is not changing as rapidly as E, whose variations probably arise therefore from changes in resistance.

The Ringer frogs have the lower conductances in each case just as they do when mounted between Ringer solutions on each side. Apart from this difference the cathodic polarizations of tap-water and of Ringer frogs are very similar. In both cases $E^0_{7\frac{1}{4}} < E^0_0$ and $E_7 > E_{\frac{1}{4}}$, but in the tap-water frogs (kept in tap-water for 7 days before killing) a slight initial fall in E from $\frac{1}{4}$ to 1 min. shows that the initial change observed in E when the skins are polarized cathodically at these current densities in Ringer solution persists with dilute external solutions. The latter remove both the positive values for $(E^0_{7\frac{1}{4}} - E^0_0)$ and the negative values for $(A_7 - A_0)$ which illustrates the correlation between these two properties even for variations of composition of the external solution from Ringer to M/500 KCl.

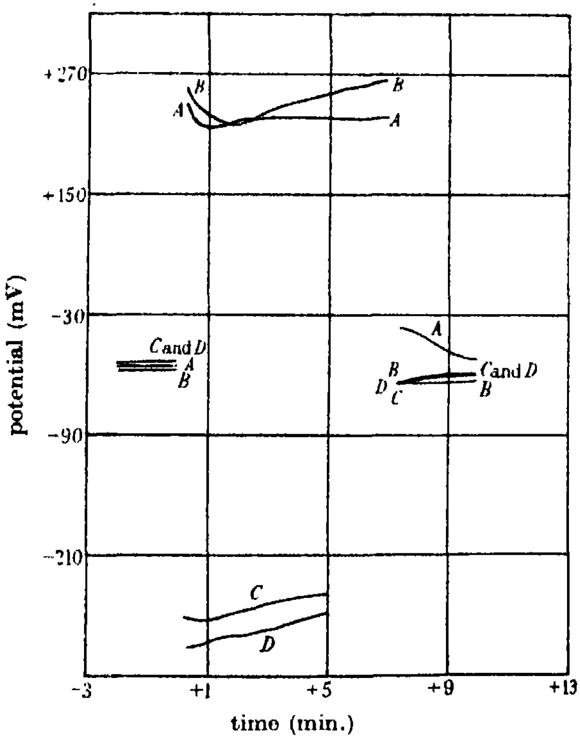

FIGURE 8

DISCUSSION OF THE ESSENTIAL FEATURES OF THE RESULTS

Any attempt to interpret the data set out above must explain three essential features which are as follows:

(i) That the polarization at constant current density over the first ¼ sec. shows that the skin has at least two time constants.

(ii) That $(E^0_{AP}-E^0_{BP})$ and $(\Lambda_{AP}-\Lambda_{BP})$ are negatively correlated for the following types of variation:

(*a*) between frogs (1 % level); (*b*) between occasions (1 % level); (*c*) frog-occasion interaction (1 % level) or for chance fluctuations not arising from variations of frog or of occasion; (*d*) current density (see for example, figure 5); (*e*) pressure gradient across the skin; (*f*) treatment of living frogs with Ringer solution prior to killing; (*g*) replacing the Ringer solution in contact with the outer side of the skin by M/500 KCl.

Since there is no significant correlation between E^0_{BP} and Λ_{BP} these correlations do not depend on any relationship between E^0 and Λ.

(iii) That for every single effect observed at least one skin on one occasion has shown a directly opposite effect. This does not apply to the case of polarizations with dilute external solutions where very few observations have been made and where every effect has been qualitatively reproducible on all the occasions tried.

The following conclusions may be drawn from these essential features:

(i) That the skin is non-homogeneous electrically in depth so that successive laminae parallel to the skin but at different depths in it are dissimilar.

(ii) That the skin is electrically non-homogeneous in directions parallel to its surface so that laminae parallel to that surface are in general mosaic rather than truly homogeneous laminae.

(iii) That the different electrical units of which the skin is composed contain between them factors making both for all effects and for their opposites; the actual state of any skin depends on a balance between all these opposing factors. If all these factors are subject to fairly large fluctuations as between frogs, successive occasions, etc., it is merely necessary to assume that these fluctuations are independent of each other in almost all cases except only in the factors leading to the strong correlation referred to above.

Any membrane can be regarded as consisting of a number of electrical units each of which is homogeneous and has its own resistance and parallel capacity whose product gives the time constant of the unit. A membrane consisting of a single layer of different electrical units will behave electrically like a single capacity in parallel with a single resistance and will therefore have a single time constant if the impedance between one unit and another is negligible, that is to say if the surface resistance of the membrane is negligible. Since Ringer solution conducts well, the resistance along the surface of frog skin is likely to be low. Thus frog skin with its two electrical time constants can hardly be treated as being electrically homogeneous throughout the whole of its depth, and the conclusion is only too obvious to anyone acquainted with the microscopic structure of frog skin. The unit corresponding to the shorter time constant will be referred to as the series resistance and it is as well to state that its resistance is too great to be accounted for by that of the Ringer solution between the skin and the measuring electrodes. There is no evidence as to the electrical homogeneity or not of the series resistance other than that it has not yet been resolved into more than one component. The evidence for this conclusion is not easily interpreted on any other hypothesis and the conclusion itself is inherently plausible.

The electrical evidence for the second conclusion is also strong but not absolutely conclusive although supported by grounds of general plausibility. Thus, for any electrolytic cell in equilibrium, the results of concentration polarization, due to the flow of polarizing current, are such that $(E^0_{AP} - E^0_{BP})$ and the polarizing current have the same sign. This follows since E^0_l must alter in such a way as to oppose the polarizing current; if it did not, fluctuations due to the thermal motions of the molecules would cause a current to flow so that the cell could never stay in that state and be in equilibrium with E^0_{BP}. In consequence membranes where $E^0_{AP} - E^0_{BP}$ and the polarizing current have opposite signs will be said to show an anti-concentration-polarization effect. The presence of such an effect indicates the existence of some form of disequilibrium. If $E^0_{AP} - E^0_{BP}$ is plotted against current density an anti-concentration-polarization effect is shown by points lying in the second and fourth quadrants rather than in the first and third quadrants. Reference to figure 5, curve A, shows all the points except that at the highest cathodic current density in the second and fourth quadrants and the result is valid to the 1 % level of significance. Thus frog skin shows the anti-concentration-polarization effect over certain ranges of current density and must therefore be in a state of disequilibrium.

Three types of disequilibrium suggest themselves in the case of frog skin. The first is a diffusion process acting at a homogeneous surface, the second is a respiratory process acting at a homogeneous surface, and the third is one or other or both of the above types of process acting at a non-homogeneous surface. The latter hypothesis will be referred to as the local action hypothesis owing to analogies with the local action currents of corroding metals. General arguments against curve A of figure 5 being explicable by either of the first two hypotheses can be given and neither of these hypotheses as such explains the correlation between $E^0_{AP}-E^0_{BP}$ and $\Lambda_{AP}-\Lambda_{BP}$ that has to be explained.

The first type of disequilibrium involves a simple diffusion potential being responsible for the electromotive force. Thus the potential E is given by

$$E=-\sum^{i}\int\frac{\mu_i}{z_iF}\left(\frac{\partial t_i}{\partial x}\right)dx,$$

where integration is round the complete circuit and all the terms have their usual thermodynamical significance. If the circuit involves one or two regions where the transport number changes very rapidly the large values for $\partial t_i/\partial x$ lead to correspondingly high values of the integrand and these terms contribute the greater part of the value of E. Big values for dt_i occur at phase boundaries rather than in bulk phases and the value depends more on change of phase than on concentration gradients. Thus electrolysis is liable to affect E more by changes in μ_i than in $\partial t_i/\partial x$ if the greater fraction of the diffusion potential is concentrated at phase boundaries. Thus the value for ΔE the change in E due to electrolysis depends mainly on

$$-\sum^{i}\int\frac{\Delta\mu_i}{z_iF}\left(\frac{\partial t_i}{\partial x}\right)dx$$

rather than on

$$-\sum^{i}\int\frac{\mu_i}{z_iF}\left(\Delta\frac{\partial t_i}{\partial x}\right)dx.$$

If ionic interaction can be neglected so that μ_i depends at a given temperature and pressure only on the solvent and on the concentration c_i of ion i it follows that

$$\Delta\mu_i=\int_0^{\Delta c_i}\frac{\partial\mu_i}{\partial c_i}dc_i,$$

and since for stability $\partial\mu_i/\partial c_i$ is positive it follows that $\Delta\mu_i/\Delta c_i$ is positive. For current flowing against E, Δc_i and $\partial t_i/\partial x$ have opposite signs so that ΔE is positive. This result shows that no anti-concentration-polarization effect is to be expected if the greater part of a diffusion potential arises from terms at phase boundaries though special cases involving weak electrolytes might sometimes show it. Observed frog skin potentials with Ringer on each side sometimes exceed 100 mV while the partition ratio of the major constituent ions of Ringer between Ringer and cell protoplasm can seldom if ever exceed 100 to 1. It seems likely therefore that phase boundary potentials are involved in skin potential, and that a simple diffusion potential across homogeneous boundaries of this nature would not lead to the marked anti-concentration-polarization effects observed.

There is no doubt that tissue respiration is necessary for the maintenance of the potential and that enzyme reactions at certain pH's are very sensitive to changes in pH. The skin potential however is hardly affected by changes of pH between 7·6 and 9·0, the tissue is weakly self buffered, and the Ringer solution is buffered to a pH of 8. Thus the theory that concentration polarization from $12\,\mu$A-cm.$^{-2}$ for 15 sec. leads to local changes of pH and that these affect respiration so as to produce an anti-concentration polarization effect is hardly tenable. Apart from hydrogen and hydroxyl ion no other ion likely to affect enzyme action to a sufficient extent seems to be available.

The arguments just given suggest that the two types of disequilibrium that have been discussed are not likely to be the explanation of the anti-concentration-polarization effect. The third alternative has already been used to account for anti-concentration-polarization effects in the case of the anodic polarization of copper in aerated solutions (Gatty and Spooner 1938). This explanation accounts for the correlation between $E^0_{AP} - E^0_{BP}$ and $\Lambda_{AP} - \Lambda_{BP}$ and is in terms of local action theory.

In its simplest form local action theory deals with a single electromotively active surface which consists of patches of two different kinds. The patches of one kind form the anodic area and of the other form the cathodic area of respective total surfaces A_A and A_C. The electrical potential of the inner side of any patch of anodic area less the potential of the outer side of that patch is denoted by E_A while the corresponding potential difference across any and every patch of cathodic area is supposed to be E_C. Current is said to be positive or anodic when it flows from an external circuit to the inner side of the electromotive surface, through the surface, and back from its outer side into an external circuit. Denoting the total current across the anodic and cathodic areas respectively by i_A and i_C, it follows that for zero external polarizing current $i_A - i_C = i$, where i denotes the local action current. Denoting the electrical conductances of the anodic and cathodic areas and their resistances respectively by Λ_A, Λ_B, R_A, and R_B it is possible to write for zero external polarizing current $E^{i'}_A = E^0_A + i'R_A$ and

$$E^{i'}_C = E^0_C - i'R_C;$$

since Ohm's law shows that E_A and E_C depend on i_A and i_C and should therefore be written $E_A^{i_A}$ and $E_C^{i_C}$ and zero polarizing current i means $i_A = -i_C = i'$.

An additional simplification follows if the value of $[R_A + R_C]$ is great compared to the remainder of the resistance in the local action circuit for in this case $E_A^{i'} = E_C^{i'} = E^0$ where E^0 is the observed potential with no external polarizing current so that

$$E^0 = \frac{\Lambda_C E_C^0 + \Lambda_A E_A^0}{\Lambda_C + \Lambda_A}. \tag{1}$$

The observed potential is then the conductance weighted mean of the unself-polarized potentials E_A^0 and E_C^0 of the anodic and cathodic areas.

Expression (1) only holds if the concentration polarization effects of local action on the values of E_A and E_C can be neglected in addition to the resistance between anodic area patches and patches of cathodic area. It illustrates however a mechanism by which E^0 can depend on resistances or conductances. It therefore gives a possible explanation of the correlation that has to be explained.

When it is realized that the immersion of a clean metal surface in an aerated solution leads to a non-homogeneous interphase which produces local action currents, it is evident that local action currents are likely to occur between small units on the surfaces of living cells which are presumably more complicated than those of solution-metal interphases. On a microscopic scale local action currents are likely owing to the cellular structure of skin and on a macroscopic scale two pieces of skin off the belly of the same frog, even when cut out symmetrically, have sometimes been observed to give potentials differing by 50 % of the bigger potential of the two. Rough (compared to the care normally employed) as opposed to destructive handling of the skins during mounting seems to make little difference to the potential. Thus the observed differences amount to a direct demonstration of local action currents on a macroscopic scale since the differences must be assumed to lie in the skin itself. Finally, the iron wire model of nervous activity behaves as it does because of local action currents and this affords an analogy between these currents and the bioelectrical behaviour of nerve. Thus the local action hypothesis must be judged very plausible on general grounds apart from expression (1) above and the difficulty of accounting in any other way for anti-concentration-polarization effects.

Thus the first two essential features show that frog skin is electrically non-homogeneous and in a state of disequilibrium; they also are presumptive evidence for a series resistance and for local action currents. The third essential feature is readily explained in terms of a non-homogeneous skin where the properties of the units can vary independently. The simplest model for the electrical behaviour of frog skin accepting these conclusions is a three-unit one as shown in figure 9.

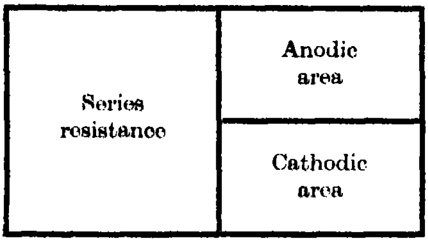

FIGURE 9

Discussion of results. The residual features

The paucity of existing data and the possible complexities of skin structure show that attempts to interpret the electrical behaviour of skins can only be tentative at this stage. A very simple model is shown in figure 9.

It is therefore of interest to show what further hypotheses have to be made for this model to account for the residual features of the polarization curves and the known data about frog skin. A self consistent picture can be obtained with the following additional hypotheses, but local action theory allows considerable elasticity of interpretation so that the following hypotheses should not be regarded as final. Arguments in favour of each of them can be deduced from the curves published in this paper together with data mentioned by Dean and Gatty 1937.

The electrical conductance of isolated frog skin with Ringer solution on both sides decreases with time for several hours after mounting and this age effect on the conductance occurs in the series resistance. The anodic area conductance is reduced by cathodic and increased by anodic polarizing currents. The cathodic area conductance is increased by cathodic and decreased by anodic polarizing currents. The anodic area conductance is more sensitive to polarizing currents than the cathodic area conductance. The cathodic area is relatively more permeable to anions on its inner than its outer side (assuming that conductance changes are less due to specific ions than to total electrolyte concentration). Dilute outer side solutions lead to a negative anodic area potential and hence the anodic area is more permeable on its outer side to cations than to anions. Dilute outer side solutions lead to a decrease in the cathodic area conductance. The series resistance lies on the inner side of the anodic and cathodic areas (dilute outer side solutions do not affect it enough for it to be accessible to them). The anodic area of frogs kept in Ringer solution is in the condition which the anodic area of frogs kept in tap water reaches after the passage of cathodic polarizing current. With dilute outer side solutions the anodic area conductance decreases with cathodic polarizing currents in the case of tap water (frogs kept in tap water but their skins mounted with Ringer in contact with its inner side), but not in the case of Ringer frogs (frogs kept in Ringer before killing); thus anodic area behaviour in essentials is unaffected by dilution of the outer side solution. With dilute outer side solutions cathode polarization leads to an increase in cathodic area conductance. The decrease in cathodic area conductance with dilute outer side solutions is due to dilution of electrolyte contained in the cathodic area. The cathodic area is readily accessible in an osmotic sense to outer side solutions and is more so than the anodic area or the series resistance. Anodic polarization of skins with dilute outer side solutions leads to an increase in anodic area conductance. Anodic polarization of skins with dilute outer side solutions leads to a decrease in cathodic area conductance. With dilute outer side solutions the anodic area conductance is more sensitive to polarizing currents than the cathodic area conductance. Anodic polarization at 25 μA-cm.$^{-2}$ with dilute outer side solutions in the case of tap-water frogs leads to an increased concentration of electrolyte in contact with the outer side of the anodic area and in consequence of this concentration polarization leads to an increased potential. The cathodic area is relatively more permeable to anions than to cations (since the chloride free effect (Dean and Gatty 1937) shows that part of the electromotively active portions of the skin is anion sensitive). Frog skins with a relatively greater pressure on the inner side of the skin have more positive anodic potentials and a lower cathodic area conductance than skins with the pressure gradient reversed. Skins with positive pressure gradients have relatively smaller areas of cathodic and relatively greater areas of anodic area in their local action surface. The cathodic area of all skins has a higher conductance cm.$^{-2}$ than their anodic areas. It is therefore possible that the cathodic area is a pore between cells.

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The fine structure of phloem fibres

II. Untreated and swollen jute

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[Plate 5]

Studies of the jute fibre by X-ray methods, under the polarizing microscope, and after swelling with various reagents, lead to the conclusion that the optical heterogeneity of the wall is not to be explained in terms of changes in cellulose chain direction. The evidence available shows that the wall is composed of chains forming a single, steep spiral. The wall spiral becomes steeper as the cell elongates and this continues until the onset of wall thickening.

The fibre in many ways provides a study complementary to that of hemp. The outer layers of the wall are the least lignified and have the least resistance to swelling. The phenomenon of 'ballooning' is therefore never observed, though structures resembling balloons may be produced by a special technique. For similar reasons transverse cracks are never observed during the swelling process.

Impregnation with lignin seems to cause an increase in thickness of the wall even in the outer layers which are far removed from the cytoplasm-wall interface.

The results fully confirm the conclusions already reached that observations of swollen material is usually entirely misleading.

INTRODUCTION

In the previous paper in this series (Kundu and Preston 1940), it was pointed out that while in hemp the wall is certainly heterogeneous in its optical properties, this does not necessarily imply that the cellulose chains in outer wall layers are less steeply inclined to the longitudinal than are those of inner layers. It is proposed here to re-examine the structure of jute fibres from this point of view. For a review of the various statements which have been made concerning the 'crossed fibrillar' structure of plant cell walls the reader is referred to previous communications (Kundu and Preston 1940; Preston 1939*a*). Here again we have to deal mainly with the cellulose component of the wall, though the various phenomena observable during swelling demand some reference to the incrusting substances.

In broad outline, the wall of the jute fibre resembles that of hemp; in particular the evidence is again convincing that there is no considerable change in cellulose chain direction from layer to layer. It is not, therefore, proposed to give any more than an indication of these points of similarity. In some ways, however, the work on jute is complementary to that on hemp and it is on this aspect that particular emphasis is placed. The details of the anatomy of the jute plant will be examined elsewhere (Kundu 1940). It will suffice here to point out (pending this later discussion) that in jute, contrary to accepted opinion, all the so-called 'bast' fibres are developed from a cambium. There seems no reason to doubt, moreover, that the first-formed, outermost fibres are developed in protophloem and the innermost in metaphloem. The terms *protophloem fibres* and *metaphloem fibres* respectively will therefore be used to denote fibres of the outer and inner series wherever necessary. It may be said at the outset that no major differences have been observed between the two groups.

In transverse section under the polarizing microscope the fibres of jute resemble those of hemp very closely, with the exception that an inner bright lamella is very seldom observed. The outer layer is, however, clearly distinct from an inner broader dark lamella. Thus in jute we have the same optical discontinuity as in many other plant cells, and again its explanation is no more easy to give.

Fibres have been observed in the untreated, water-retted, and chromic acid macerated state. The methods employed in treating the material are identical with those described in the previous paper and no further reference need be made to them. The material used was obtained from plants grown at Kew, rather than from commercial fibre, since this work on the wall was coupled with an anatomical investigation of the jute plant. The measurements of wall thickness (table 2) and the earlier determinations of the major extinction position were carried out by Dr Kundu under the direction of the writer, but the latter have all been revised before publication.

CELLULOSE-CHAIN DIRECTION AND WALL LAYERING

Although the results of major extinction position measurements in jute fibres are essentially similar to those already published for hemp, the comparative clarity with which two lamellae can be distinguished under the polarizing microscope makes it advisable to pay more attention to them than they might otherwise merit. The extinction positions have been measured on cells cut open as described elsewhere (Preston 1934) both for protophloem and metaphloem fibres with the wall fully thickened and only partially so, and a representative set of data are presented in table 1. It is implicitly assumed in what follows that the figures for the partially thickened fibres represent an early condition of the fully thickened. It may be said immediately that this is not definitely proved. We cannot observe one fibre in each of the two conditions, and in the case of protophloem fibres it is impossible to compare two sets even at the same level in the stem. Nevertheless, observations over a wide range of material has always yielded results of the type presented. The assumption we make would seem to be justified.

TABLE 1. THE INCLINATION TO THE LONGITUDINAL OF THE M.E.P.S OF FIBRES FULLY THICKENED AND PARTIALLY THICKENED

r. = right-hand spiral. l. = left-hand spiral.
Averages are of 50 determinations each.

		major extinction position (degrees)	
fibre type	condition	range*	average†
metaphloem	thickened	23·0–0·0	7·9
	partially thickened	22·0–2·0	7·4
protophloem	thickened	17·5–0·0	5·5
	partially thickened	14·0–0·0	5·7

* The m.e.p. is usually inclined in a right-handed spiral; only occasionally is a left-hand spiral encountered.

† The distribution is markedly skew, the modal value lying between 0° and 5° in each case. Hence a probable error cannot be given.

Measurement of layer thickness, both under the ordinary and polarizing microscope, are presented in table 2. These were made in the ordinary way using a $\frac{1}{12}$ in. oil immersion lens, with a Leitz eyepiece screw micrometer

TABLE 2. LAYER THICKNESS IN FIBRES FULLY AND PARTIALLY THICKENED

		thickness		
fibre type	condition	whole wall*	inner layer†	outer layer‡
protophloem	thickened	4·30 ± 0·09	3·87 ± 0·09	0·42 ± 0·04
	partially thickened	1·38 ± 0·05	1·19 ± 0·04	0·18 ± 0·04
metaphloem	thickened	3·81 ± 0·16	3·22 ± 0·16	0·60 ± 0·10
	partially thickened	2·12 ± 0·07	1·68 ± 0·11	0·44 ± 0·05

* Under the ordinary microscope.
† Under the polarizing microscope (dark).
‡ Under the polarizing microscope (bright).

clamped firmly to the microscope. While such determinations cannot be said to have a high order of precision, the differences which we observe are well outside the limits of experimental error. Several points emerge immediately. In agreement with the results of Bailey and Kerr (1935) on the tracheid, increase in wall thickness is here also due largely to increase in the inner dark layer. We have, however, the astonishing fact that during differentiation the outer layer too thickens appreciably. This cannot reasonably be explained in terms of intussusception in the accepted sense, for the process occurs long after the development of an interface between protoplasm and wall; nor does it seem likely that the effect is due to a progressive change of the cellulose chain direction in outer lamellae of the *inner* layer so that they become effectively part of the *outer* layer. The cells at the younger stage, however, show little signs of lignification and in this respect two possibilities are immediately obvious. The process of lignification either causes a morphological layer already present to swell considerably or, as inner lamellae continue to lignify, simply adds to the thickness of an outer 'layer' characterized solely by its chemical complexity. The former alternative seems more feasible in view of the repeated observation that lignification causes increase in wall thickness (e.g. Frey 1926; Alexandrow and Djaparidze 1927).

The main interest of the figures for wall thickness, however, centres round their relation to the m.e.p. determinations. As we pointed out in the case of hemp, close agreement between the directions of the m.e.p.s for fully and slightly thickened fibres furnishes strong evidence against the existence of approximately transverse cellulose chains in the outer layers of the wall. Only if the cellulose chains in outer and inner layers were *exactly* at right angles could this agreement hold. In practice, however, whenever the angle between fibrils has been measured in a wall possessing such a crossed structure, the interfibrillar angle has been found considerably less than 90° (Preston and Astbury 1937; Astbury and Preston 1940; Preston 1939*b*; Steinbrinck 1927). This would mean that if one layer were increased in thickness the direction of the m.e.p. would change. In the present circumstances the differences in wall thickness seem to be sufficient, if the wall really were of a crossed fibrillar nature, to cause a change so pronounced as to be measurable. There is no need to give here the calculations involved, but it appears that if the reasonably high angle of 80° be assumed then the change in the m.e.p. would be some 2° or 3°, assuming a birefringence of 0·06. This rough figure is calculated on the assumption that the thickness of the outer layers represents real differences in the amount of cellulose present, and is therefore a minimum; thus the most unfavourable conditions give a measurable difference. In table 1, it will be observed that there is no difference of this magnitude.

Thus in jute, as in hemp, consideration of the m.e.p. argues strongly against the presence of an outer layer with more or less transverse cellulose chains. The simplest and most logical conclusion is that only one set of chains is present and that the optical discontinuity of the wall is due to some cause other than change in chain direction.

This conclusion receives full support from the X-ray diagram. Figure 7, plate 5 represents the diagram of a bundle of jute fibres containing both protophloem and metaphloem groups. Neither in our material nor in commercial jute* is there any sign of a cellulose chain orientation other than longitudinal or steeply spiral.

STAINING REACTIONS OF THE WALL AND THE EFFECT OF SWELLING AGENTS

A rough estimation of the distribution of the incrusting substances has been made in fibres from untreated, water-retted, and chromic-macerated material. In the first condition only were the fibres observed in transverse section. Staining methods were used exactly as in the case of hemp. They reveal, however, a distribution, particularly of lignin, significantly different from that in hemp. In both protophloem and metaphloem fibres the wall is strongly impregnated with lignin in a fairly uniform manner, lignification in the former being somewhat less marked. Certainly the degree of lignification seems to be higher than in hemp and in this our staining methods are supported by the analyses of Norman (1936). Pectin also is distributed throughout the whole wall, though it is much more abundant in the middle lamella.

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* The statement concerning the commercial fibre was communicated privately by Dr W. T. Astbury.

After retting in water, the primary wall and the outer layers of the secondary wall lose much of their lignin, while the inner layers remain strongly lignified; pectin has disappeared completely. Maceration in chromic acid removes all lignin from the wall as far as can be ascertained by staining methods.

The distribution of incrusting substances is thus very different from that in the hemp fibre, where the outer wall layers are the more strongly lignified, and retain much of their lignin even after water-retting. We might, therefore, expect a response to swelling agents which, though similar in general outline, will be different in detail. This expectation is fully confirmed. We will confine ourselves to water-retted fibres since they show the most striking differences. The innermost layers of the wall, which are still lignified, fail to dissolve or even swell appreciably in any strength of sulphuric acid; neither do they swell in caustic soda or in Eau de Javelle. In all these treatments the layers of the wall outside this resistant layer swell considerably (and even dissolve completely) in sulphuric acid in strengths above 70 %. The outermost layer of all, possibly the primary wall, usually has the least resistance to swelling though in certain cases it may temporarily retain a consistency sufficient to produce signs of constrictions in the swelling of inner layers. In sulphuric acid of more than 50 % concentration this layer swells and breaks up into fibrils—a change which also occurs if the wall is compressed after a 36-hour treatment in the cold with Eau de Javelle or 10 % caustic soda. Under these latter conditions the fibrils take the form of comparatively flat spirals which become flatter as greater pressures are applied (table 3 and figures 4–6, plate 1).

TABLE 3. INCLINATION OF FIBRILS TO THE LONGITUDINAL IN FIBRES TREATED WITH EAU DE JAVELLE. CAUSTIC SODA (10 %) AND SULPHURIC ACID PRODUCE SIMILAR EFFECTS

	inclination (degrees)		
fibre	gentle pressure	medium pressure	violent pressure
1	14·0	22·0	27·0
2	12·5	24·0	36·0
3	18·0	25·0	30·0
4	18·0	21·0	35·0
5	14·0	22·0	32·0
6	9·0	20·0	26·0
7	14·5	25·5	33·5
8	19·0	24·5	41·0
9	15·5	24·5	38·0
10	18·5	23·5	27·0

At the same time the slit mouths of the pits always so orient themselves that they lie parallel to the fibrillar direction in the outer layers of the wall (figures 1–3, plate 5). It may be mentioned that fibrils are never seen in the inner layers even after the highest pressures. We have therefore here again the clearest evidence that the appearance, after swelling, of spiral fibrils is an artefact solely attributable to the effect of swelling agents and subsequent treatment upon the dimensions of the fibre. Such pictures of the wall after swelling, as produced for example by Osborne (1935), are merely a consequence of the deformation of an originally steep spiral and give a totally misleading picture of the structure of the intact cell.

In view of the connexion suggested elsewhere between the phenomenon of 'ballooning' and the resistance of an outer layer to swelling, it is interesting to note that in jute this phenomenon is seldom observed. This would seem to be connected with the lower lignin content of the outer layers in the jute fibre wall. During the present investigation special attempts have been made to induce ballooning in jute in the light of our knowledge of the behaviour of hemp fibres. After treatment with 55% sulphuric acid the fibres certainly swell, but no splitting of the primary wall takes place. This primary wall may still be observed sheathing the wall as a system of spiral fibrils. The phenomenon both in appearance and development is quite distinct from ballooning proper. Under special conditions, however, a phenomenon may be observed which is much more comparable with ballooning and is developed in the same way. If water-retted fibres are treated with Eau de Javelle for 36 hours, washed in water, immersed in 55% sulphuric acid, and compressed, they present the appearance illustrated in figure 8, plate 5. Even here the ballooning can hardly be described as typical, for the 'balloons' appear to be flattened discs rather than spheres. This particular treatment has obviously 'softened' the primary wall sufficiently to allow it to split rather than disorganize into fibrils, and at the same time has allowed the inner layers to swell to an extent such that they protrude through the spiral fissure. Upon further pressure being applied to fibres in this condition, the outer layers become entirely disorganized and the appearance of balloons is lost.

In jute again, as in hemp, transverse markings often make their appearance on swelling, particularly if pressure is employed. Only under the conditions of slightest pressure or if the swelling is unusually great are the transverse markings not evident. Here we have the clearest evidence that these markings are folds in the wall and not striations (figure 9, plate 5). This lends further support, if any were needed, to similar conclusions in the case of hemp.

The development of the fibre

While in this paper we are primarily concerned with the structure of the adult wall, some brief remarks on the nature of the wall at periods during the development of the fibre may perhaps not be out of place, particularly since the point has been referred to elsewhere (Barker 1940). Further details concerning the botanical side of the process are to be published later (Kundu 1940). We may merely note that the young fibre is enveloped in a wall which is denoted as primary and whose surface area increases during the considerable elongation of the differentiating cell. The wall appears to be pitted at a very early stage, and during the whole process of elongation the direction of the m.e.p. corresponds, within the limits of experimental error, with that of the major axis of the pit mouth. Up to a fairly late stage in development the pit mouth is broadly elliptical and both the m.e.p. and the major axis of the pit mouth make a considerable angle to the longitudinal. When, however, the fibre has reached its full length, the pit has become slit-mouthed, and the inclination of the slit is more nearly longitudinal. No fibre has yet been observed in which the pit mouth is oriented transversely. Thus we have yet another clear case in which the cellulose chains of the primary wall run in a spiral whose inclination to the longitudinal decreases as the spiral elongates.

There is no point in attempting here any numerical relation between inclination of the pit mouth and cell length. The appearances suggests that during the major part of cell extension the direction both of the m.e.p. and the slit mouths is maintained without change, and that it is the last phase only of extension which is effective in steepening the spiral. This is a point which it will be difficult to establish, since it is at present not feasible to observe a series of stages of fibre development in radial seriation. It does recall the differences between the effects of wall growth and of mechanical extension suggested elsewhere for different cell types (Preston 1934, 1939 *a* and *b*).

Discussion and conclusions

Thus in jute, as in hemp, the simplest picture of the structure of the fibre wall, and one which is in harmony with all known details, is that the cellulose chains composing the wall lie in one direction only, forming a single steep spiral round the cell, and that this spiral results from an original flatter spiral as a result of cell extension. In support of this statement we have the fact that the m.e.p. of fully thickened fibres is not more steeply inclined than in partly thickened fibres as the presence of an outer layer with transverse chains would require. Again, the X-ray diagram shows most clearly that any chains with orientation other than the longitudinal are negligible in amount. Finally, the outer layers of the wall break up under the effect of swelling agents into fibrils which are first arranged in a very steep spiral, and it is only after drastic treatment of either a chemical or mechanical nature that these outer fibrils come to be arranged even approximately in the transverse direction.

Perhaps the most important conclusions to be drawn centre around the connexion between the effect of swelling agents and the distribution of incrusting substances. This is hardly the place to refer to details of jute manufacture, but it would certainly seem that the lower quality of jute as a textile fibre may be largely attributable not only to the high xylan content (Norman 1936) but also to its characteristic lignin distribution. The tendency of jute to 'linter' for instance may well be due to the less resistance of the outer layers of the wall in water-retted material. The absence of typical 'ballooning' is certainly connected with the lack of an outer resistant sheath, and to the comparative uniformity of swelling in inner layers is due the fact that no such transverse cracks have been observed as were so very evident in hemp. The frequent presence of folds in outer layers of swollen material, on the other hand, has been observed, both in jute and hemp, and these folds are even more pronounced in jute. The presence of an outer non-swelling layer, in terms of which the presence of folds in hemp fibres were explained, is evidently not essential from this point of view. It is, in fact, difficult to say why folds are produced in jute fibres. Pressure on the material is certainly required, and it seems certain that there must therefore be some difference between the responses of inner and outer layers to a combination of pressure and swelling, which is not attributable to the swelling responses only.

In both jute and hemp fibres, then, the wall is uniform in the sense that the cellulose chain direction is maintained throughout the wall thickness without serious change. The effects of swelling agents in both cases depends not only on the precise configuration of the cellulose component itself but also on the amount and distribution of the incrusting substances. In both cases the evidence points to the development of the fibre from an originally spirally wound initial, the inclination of whose wall spiral changes in accordance with change in dimension at some stage of growth. The optical heterogeneity still awaits explanation. The suggestion already made for hemp that it may be connected with the incrusting complex has perhaps received further support from jute in the observation that the outer layer, bright in cross-section, becomes considerably thicker during lignification. It would, however, seem more likely that the optical properties are connected primarily with the cellulose component and change only slightly with the development of incrusting substances.

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DESCRIPTION OF PLATE 5

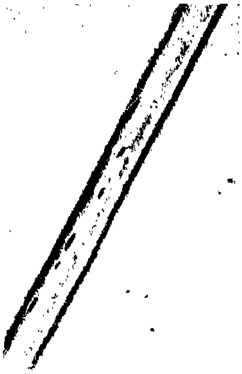
FIGURES 1–3. Adult fibres of *Corchorus*. FIGURE 1. Untreated. Note the pits with slit mouths inclined in a steep spiral. FIGURE 2. After treatment with 52·5 % sulphuric acid followed by pressure. The pit mouths are now less steeply inclined. FIGURE 3. As in figure 2 after violent pressure. The inclination of the slit mouths to the cell axis is now much greater.

FIGURES 4–6. Illustrations of the progressive change in fibrillar direction in *Corchorus* fibres after treatment with Eau de Javelle and various pressures. FIGURE 4. Gentle pressure. FIGURE 5. Strong pressure. FIGURE 6. Violent pressure.

FIGURE 7. X-ray photograph of a bundle of jute fibres. The X-ray beam is perpendicular to the longitudinal axis of the fibres.

FIGURE 8. 'Ballooning' in *Corchorus* fibres after immersion for 36 hours in Eau de Javelle followed by 55 % sulphuric acid and compression.

FIGURE 9. Transverse markings on swollen fibres. The wrinklings at the edges show clearly that these are folds and not striations.



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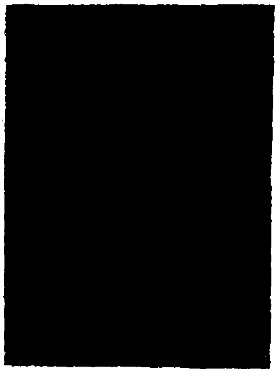

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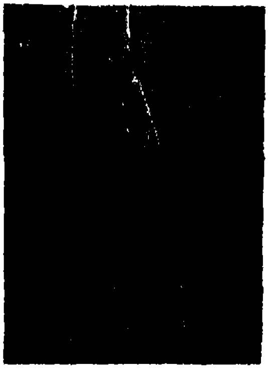

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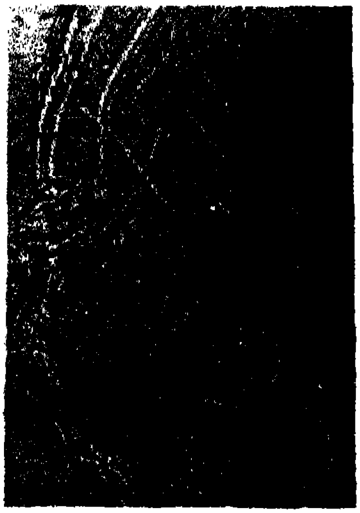

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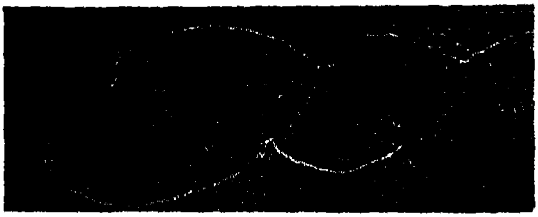

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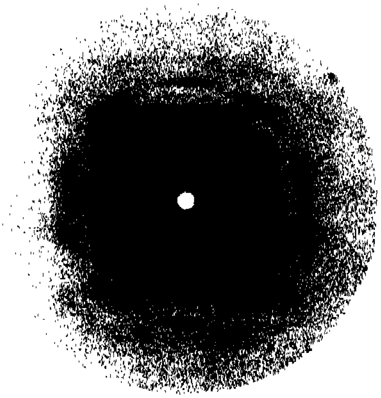

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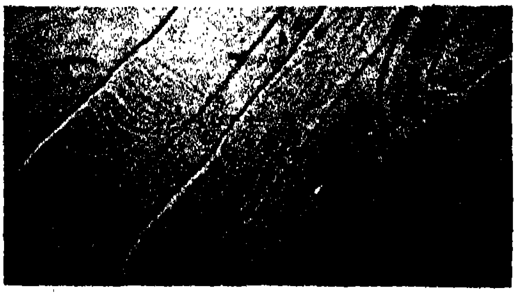

9

A survey of anthocyanins

VII. The natural selection of flower colour

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The anthocyanins have been identified in the flowers, fruits or leaves of approximately 200 species of plants. The results have been combined with earlier data, to ascertain the frequency with which derivatives of the three main anthocyanidin types occur as flower pigments among the species so far examined.

Classification of the natural habitats of the species examined shows that pelargonidin derivatives predominate in the flowers of tropical and subtropical species, while delphinidin derivatives are the commonest in temperate and alpine plants.

The colours of tropical and subtropical flowers containing cyanidin or delphinidin derivatives are generally redder than those of temperate species containing the same anthocyanin. It is concluded that red-flowered forms have a greater survival value than blue in most tropical plants.

On the basis of the anthocyanin present in the flowers of thirty-two species of *Tulipa*, the genus falls into two groups in accordance with the morphological classification.

INTRODUCTION

Of 832 dicotyledonous species in the British Flora with conspicuous, coloured flowers (white, yellow or cyanic), 407 (49 %) have anthocyanins in the flowers of either the wild type or at least of naturally occurring varieties. These anthocyanins are of several kinds, and are derived from three anthocyanidins: pelargonidin, with one hydroxyl group in the 2-phenyl ring; cyanidin, with two hydroxyl groups in the 2-phenyl ring; delphinidin, with three hydroxyl groups in the 2-phenyl ring. The presence of one of these types rather than another must depend on either its chemical character (i.e. its mode of synthesis and chemical properties) or its biological value. A study of the distribution of the various anthocyanins throughout the flowering plants should help to decide which is the effective relationship. Such a survey was initiated by Robinson and Robinson in 1931.

It included fruits and leaves in addition to flowers, and the results of Parts I–VI have recently been summarized together with fresh data by Lawrence, Price, Robinson and Robinson (1939).

We have now identified the anthocyanins of a further number of flowers, fruits and leaves and the new facts (tables 1, 3 and 4) are set out in a form similar to that of the summary. In particular, we have identified the anthocyanins present in the flowers of thirty-two species of *Tulipa*, in order to supplement the extensive cytological and morphological studies on the genus, carried out at this institution (Upcott and La Cour 1936; Hall 1940).

TABLE 1. ANTHOCYANINS PRESENT IN FLOWERS

Note. The figures given in tables 1, 2 and 4 refer to the number allotted by Hutchinson in his classification (1926), the first indicating the order and the second the family, e.g. 76–264, order Lamiales, family Labiatae. For the terminology employed in describing glycosidic types, see Robinson and Robinson (1932). A = acylated.

DICOTYLEDONS

LAMIALES	
LABIATAE (76–264)	
Ajuga reptans var. (pink)	Cyan. 3 : 5-di. A.
Hyssopus officinalis	Delph. 3-mono.
Hyssopus officinalis var. (pink)	Pel. 3-mono.
Salvia Horminum (bracts)	Malv. 3-mono.
Salvia Horminum var. (pink bracts)	Peon. 3-mono.
Salvia splendens var. (purple)	Delph. 3 : 5-di. A.
Scutellaria costaricana	Pel. 3-mono.
VERBENACEAE (76–263)	
Clerodendron splendens	Pel. ? sugar (high distribution)
Clerodendron Thomsonae	Pel. 3-mono. (? A.)
Congea tomentosa	Cyan. 3-PG. + Cyan. 3-bio.
Verbena platensis	Delph. 3 : 5-di.
PERSONALES	
ACANTHACEAE (75–259)	
Jacobinia carnea	Cyan. 3-PG. + some Cyan. 3-bio.
Jacobinia coccinea	Cyan. 3 : 5-di. A.
Ruellia rosea	Pel. 3 : 5-di.
BIGNONIACEAE (75–257)	
Bignonia magnifica	Cyan. 3-mono.
Eccremocarpus scaber var. *aureus*	Cyan. 3-PG.
Incarvillea brevipes	Cyan. 3-bio.
Spathodea campanulata	Pel. 3-PG. + some Pel. di.
Tecoma radicans	Cyan. 3-PG.

GESNERIACEAE (75–256)	
Columnea gloriosa	Pel. 3-bio.
Streptocarpus cyaneus	Malv. 3 : 5-di.
Streptocarpus Galpinii	Malv. 3 : 5-di.
Streptocarpus grandis	Malv. 3 : 5-di.
Streptocarpus Haygarthii	Malv. 3 : 5-di. + some Pet. and/or Delph.
Streptocarpus Holstii	Malv. 3 : 5-di.
Streptocarpus Kirkii	Malv. 3 : 5-di. + some Pet. and/or Delph.
Streptocarpus Michelmorei	Malv. 3 : 5-di.
Streptocarpus nobilis	Malv. 3 : 5-di.
Streptocarpus polyanthus	Malv. 3 : 5-di. + some Pet. and/or Delph.
Streptocarpus saxorum	Delph. 3 : 5-di. + some Pet. and Malv.
SCROPHULARIACEAE (75–252)	
Angelonia salicariaefolia	Malv. 3-PG.
Antirrhinum majus var. (Scott-Moncrieff 1936)	Pel. 3-PG.
Antirrhinum speciosum	Pel. 3-mono.
Calceolaria picta	Delph. 3-PG. + some Cyan. 3-PG.
Linaria alpina	Delph. 3 : 5-di.
Linaria alpina var. (pink)	Pel. 3 : 5-di.
Linaria purpurea var. (pink)	Pel. di.
Mimulus glutinosus	Pel. 3-PG.
Tolmeia Menziesii	Malv. 3-PG.
Verbascum Blattaria	Cyan. 3-PG.
Verbascum phoeniceum vars. (purple, blue, buff)	Peon. 3-mono.
Verbascum phoeniceum var. (pink)	Pel. 3-mono.
Veronica spicata	Delph. 3 : 5-di.
Veronica spicata var. (pink)	Pel. di.

SOLANALES

SOLANACEAE (74–250)	
Iochroma tubulosa	Cyan. 3:5-di. + some Malv. 3:5-di.
Petunia integrifolia	Malv. 3:5-di. (*ca.* 60%) + Malv. 3-PG. containing some Pet. and Delph. PGs.
Streptosolen Jamesonii	Cyan. 3-bio.

BORAGINALES

BORAGINACEAE (73–249)	
Cynoglossum officinale	Delph. 3-PG. + some Delph. di.
Echium vulgare	Delph. 3 : 5-di.
Lithospermum fruticosum	Delph. di. + some Cyan. di.
Pulmonaria officinalis	Malv. 3 : 5-di. + some Pet. or Delph. di.

POLEMONIALES

POLEMONIACEAE (72–247)	
Gilia tricolor	Delph. 3 : 5-di.
Gilia tricolor var. (pink)	Cyan. 3-mono.
Phlox camlaensis (? Hort)	Delph. 3 : 5-di.
Phlox Drummondii	Malv. 3 : 5-di.
Phlox Drummondii var. (scarlet)	Cyan. 3-mono.

CAMPANALES	
CAMPANULACEAE (71–243)	
Campanula Medium	Delph. di.
Wahlenbergia graminifolia	Delph. 3 : 5-di.
PRIMULALES	
PRIMULACEAE (69–240)	
Primula farinosa	Malv. di.
Primula Forbesii var.	Malv. 3 : 5-di.
Soldanella alpina	Delph. 3-PG.
GENTIANALES	
GENTIANACEAE (68–239)	
Gentiana verna	Delph. 3 : 5-di.
ASTERALES	
COMPOSITAE (67–238)	
Gazania Pavonia	Cyan. 3-mono.
RUBIALES	
CAPRIFOLIACEAE (66–233)	
Abelia floribunda	Cyan. 3 : 5-di.
Diervilla coraensis var. *grandiflora*	Cyan. 3-PG.
APOCYNALES	
APOCYNACEAE (65–230)	
Vinca minor	Delph. 3 : 5-di.
LOGANIALES	
OLEACEAE (64–229)	
Jasminum Beesianum	Cyan. 3-PG. + some Cyan. di.
ERICALES	
EPACRIDACEAE (60–217)	
Epacris obtusifolia	Cyan. 3-mono.
VACCINIACEAE (60–216)	
Pentapterygium serpens	Cyan. 3-mono.
ERICACEAE (60–215)	
Erica cinerea	Cyan. 3-PG.
Rhododendron Fargesii	Cyan. 3-mono. + Cyan. 3 : 5-di.
Rhododendron praecox	Malv. 3 : 5-di.
RHAMNALES	
RHAMNACEAE (54–190)	
Ceanothus floribundus	Delph. 3-bio.
Ceanothus rigidus	Pet. 3-bio.
HAMAMELIDALES	
HAMAMELIDACEAE (42–151)	
Hamamelis japonica var. *rubra* (calyx)	Malv. 3-bio. + some Cyan.

LEGUMINOSAE

Papilionaceae (41–148)

Cytisus purpureus var. *incarnatus*	Cyan. 3-mono.
Indigofera Potaninii	Malv. 3-bio. + some Cyan. and Delph. (or Pet.)
Kennedya rubicunda	Pel. 3-mono.
Lotus Bertholetii	Cyan. 3-bio.
Pisum sativum var.	Malv. 3 : 5-di.
Pisum sativum var.	Cyan. 3 : 5-di. + trace Peon. or Pel.
Swainsona coronillaefolia	Delph. 3 : 5-di.
Trifolium pratense vars.	Malv. 3-PG.
Trifolium pratense vars.	Cyan. 3-PG. + little Peon. 3-PG.

Caesalpiniaceae (41–146)

Amherstia nobilis	Pel. 3-PG.
Bauhinia purpurea	Malv. 3-mono. + little Malv. di.
Cercis siliquastrum	Malv. di.
Poinciana regia	Cyan. 3-PG. + some Cyan. 3-bio.

ROSALES

Calycanthaceae (40–145)

Calycanthus floridus	Cyan. 3-mono.

Rosaceae (40–143)

Cotoneaster horizontalis	Cyan. 3-mono.
Malus atrosanguinea	Cyan. 3-mono.
Malus Eleyi	Cyan. 3-mono.
Poterium tenuifolium	Cyan. 3-mono. + some Cyan. di.
Prunus cerasifera var. *Moseri*	Cyan. 3-PG.
Prunus triloba var. fl. pl.	Cyan. 3-PG.

CUNONIALES

Grossulariaceae (39–141)

Ribes speciosum	Cyan. 3-PG.

MALPIGHIALES

Malpighiaceae (37–133)

Malpighia coccigera	Cyan. 3-(? sugar)

MALVALES

Malvaceae (36–132)

Abutilon striatum var. *Thompsonii*	Cyan. 3 : 5-di.
Callirhoë involucrata	Malv. di.
Malva moschata	Malv. 3-PG. and some Malv. di.
Pavonia multiflora	Cyan. 3-bio.

MYRTALES

Melastomaceae (33–120)

Osbeckia aspera	Malv. 3 : 5-di.

Myrtaceae (33–118)

Leptospermum scoparium var. *Nichollsii*	Cyan. 3-mono.

CUCURBITALES	
BEGONIACEAE (30–104)	
Begonia Froebelii	Cyan. 3-PG.
PASSIFLORALES	
PASSIFLORACEAE (29–101)	
Passiflora coerulea	Malv. 3-PG. (*ca.* 60%) + Malv. 3 : 5-di., both fractions containing some Pet. and/or Delph.
TAMARICALES	
TAMARICACEAE (28–98)	
Tamarix parviflora	Cyan. 3-mono. + Cyan. 3-bio.
THYMELEALES	
THYMELEACEAE (22–81)	
Daphne Mezereum	Cyan. 3-mono.
LYTHRALES	
ONAGRACEAE (21–77)	
Oenothera organensis (old fls.)	Cyan. 3-mono.
GERANIALES	
BALSAMINACEAE (20–71)	
Impatiens Balsamina var.	Malv. 3-mono.
Impatiens Balsamina var.	Peon. 3-mono.
Impatiens Balsamina var.	Pel. 3-mono.
Impatiens flaccida	Peon. 3-mono. + some Peon. 3-bio.
CARYOPHYLLALES	
CARYOPHYLLACEAE (17–53)	
Dianthus caryophyllus	Cyan. 3 : 5-di.
Dianthus caryophyllus var.	Cyan. 3-mono.
Dianthus caryophyllus var.	Pel. 3 : 5-di.
Dianthus caryophyllus var.	Pel. 3-mono.
Dianthus neglectus	Cyan. 3-bio.
Silene virginica	Cyan. 3 : 5-di.
SAXIFRAGALES	
SAXIFRAGACEAE (14–47)	
Saxifraga ligulata var. *speciosa*	Malv. 3 : 5-di. + some Pet. or Delph.
Saxifraga oppositifolia	Malv. 3 : 5-di.
VIOLALES	
VIOLACEAE (12–40)	
Viola tricolor	Delph. 3 : 5-di.

RANALES

Ranunculaceae (4–15)	
Anemone blanda	Delph. 3 : 5-di.
Anemone Hepatica	Delph. 3 : 5-di.
Aquilegia vulgaris	Delph. 3 : 5-di.
Delphinium Ajacis	Delph. 3 : 5-di.
Delphinium Ajacis var. pink	Pel. di.
Paeonia albiflora vars. (cf. Hayasi 1939*a*)	Peon. di.
Paeonia albiflora var.	Peon. di. + some Peon. mono.
Paeonia anomala	Peon. di. (*ca.* 60 %) + Peon. 3-mono. with trace of Cyan. in each fraction
Paeonia arietina var.	Peon. di.
Paeonia arietina var.	Peon. di. + some Peon. mono.
Paeonia Broteri	Peon. di.
Paeonia mollis	Peon. di.
Paeonia officinalis vars.	Peon. di. + some Peon. 3-mono.
Paeonia triternata	Peon. di.
Paeonia Veitchii	Peon. di.
Thalictrum aquilegifolium	Cyan. 3-bio.

MONOCOTYLEDONS

IRIDALES

Iridaceae (94–307)	
Gladiolus byzantinus	Malv. 3-bio.
Lapeyrousia grandiflora	Cyan. 3-PG.
Tritonia fenestrata	Pel. 3-PG.
Watsonia marginata	Malv. 3 : 5-di.

ALSTROEMERIALES

Alstroemeriaceae (90–299)	
Alstroemeria Pelegrina	Cyan. 3-mono.

LILIALES

Trilliaceae (89–295)	
Trillium sessile var. *rubrum*	Cyan. 3-PG. (*ca.* 60 %) + Cyan. di.
Liliaceae	
Aphyllanthes monspeliensis	Delph. 3 : 5-di.
Bulbocodium vernum	Cyan. 3 : 5-di.
Erythronium Hendersonii	Pet. 3-bio.
Fritillaria coccinea	Cyan. 3-PG. + little Cyan. di.
Fritillaria Meleagris	Cyan. 3-PG.
Fritillaria pyrenaica	Cyan. 3-bio. A.
Lilium Willmottiae and var. *unicolor*	Cyan. 3-PG. + some Cyan. di.

LILIACEAE (*continued*)	
Tulipa spp., Leiostemones:	
Tulipa stellata var. *chrysantha*	Pel. 3-PG. + Cyan. 3-PG.
Tulipa Clusiana	Pel. 3-PG. + Cyan. 3-PG.
Tulipa linifolia	Pel. 3-PG.
Tulipa Maximowiczii	Pel. 3-PG.
Tulipa montana	Pel. 3-PG.
Tulipa montana var.	Pel. 3-PG. + little Cyan. 3-PG.
Tulipa armena	Cyan. 3-PG.
Tulipa rhodopea	Pel. 3-PG. + Cyan. 3-PG.
Tulipa Didieri	Cyan. 3-PG. + Pel. 3-PG.
Tulipa oculus-solis	Pel. 3-PG. + Cyan. 3-PG.
Tulipa Julia	Pel. 3-PG. + Cyan. 3-PG.
Tulipa Stapfii	Pel. 3-PG.
Tulipa undulatifolia	Pel. 3-PG. + Cyan. 3-PG.
Tulipa praecox	Cyan. 3-PG. + Pel. 3-PG. + some Cyan. di.
Tulipa aleppensis	Pel. 3-PG. + Cyan. 3-PG. + some Cyan. di.
Tulipa Veneris	Pel. 3-PG. + Cyan. 3-PG. + some Cyan. di.
Tulipa kuschkensis	Pel. 3-PG.
Tulipa lanata	Pel. 3-PG.
Tulipa Hoogiana	Pel. 3-PG.
Tulipa sovietica	Pel. 3-PG. + Cyan. 3-PG.
Tulipa Eichleri	Pel. 3-PG.
Tulipa Fosteriana	Pel. 3-PG.
Tulipa Kaufmanniana	Pel. 3-PG. + some Pel. di.
Tulipa praestans	Pel. 3-PG.
Tulipa Ostrowskiana	Pel. 3-PG. + trace Cyan.
Tulipa Schmidtii	Pel. 3-PG. + Cyan. 3-PG.
Tulipa Sprengeri	Cyan. 3-PG. + some Cyan. di.
Eriostemones:	
Tulipa violacea	Cyan. 3-PG.
Tulipa pulchella	Delph. 3-PG. + Cyan. 3-PG.
Tulipa humilis	Delph. di.
Tulipa Aucheriana	Delph. di. + Delph. 3-PG.
Tulipa Bakeri	Delph. di.
Tulipa saxatilis	Delph. di.

COMMELINALES

COMMELINACEAE (84–280)	
Tradescantia bracteata, magenta var.	Cyan. 3-bio.
Tradescantia bracteata, blue var.	Cyan. 3-bio. + some Delph. 3-bio.

The earlier list contained one error and one omission which are corrected in the present list. *Pentapterygium serpens* was listed with the Ericaceae instead of under Vacciniaceae. The Leguminosae were undivided: *Amherstia nobilis* and *Cercis siliquastrum* belong to the Caesalpiniaceae, the rest of the species previously examined fall into the Papilionaceae.

The new results include varieties of species of which the wild type had previously been examined, and the wild types of certain species of which varieties had already been tested. An example is *Anemone Hepatica*—the common blue type undoubtedly contains a delphinidin diglycoside, and Schmid and Körperth (1936) must have worked with the pink variety.

Tables 2 and 3 contain the new facts on permanently pigmented leaves and fruits, together with some miscellaneous items. In each case cyanidin derivatives predominate.

TABLE 2. ANTHOCYANINS PRESENT IN PERMANENTLY PIGMENTED LEAVES

DICOTYLEDONS

75–259	*Fittonia Verschaffeltii*	Pel. 3-mono.
74–251	*Cuscuta epithymum* (stems)	Cyan. 3-mono. + some Cyan. 3-bio.
71–244	*Lobelia fulgens* var.	Cyan. 3 : 5-di. + Cyan. 3-mono.
60–215	*Calluna vulgaris* var. *cuprea*	Cyan. 3-mono.
	Gaultheria procumbens var.	Cyan. 3-mono.
50–167	*Ficus elastica*	Cyan. 3-PG.
40–143	*Spiraea venusta* var. *magnifica*	Cyan. 3 : 5-di.
35–128	*Vallea stipularis*	Delph. 3 : 5-di.
33–118	*Leptospermum pubescens*	Cyan. 3-mono.
	Leptospermum scoparium var. *Nichollsii*	Cyan. 3-mono.
20–67	*Oxalis Ortgiesii*	Malv. 3 : 5-di.
17–53	*Saponaria ocymoides*	Cyan. 3-bio.
8–32	*Papaver alpinum* (bases)	Cyan. 3-bio. + some Cyan. 3-PG.
4–15	*Paeonia Cambessedesii*	Peon. 3 : 5-di.

MONOCOTYLEDONS

105–332	*Oplismenus compositus* var. *vittatus*	Cyan. 3-(? sugar). A.
91–302	*Arum maculatum*	Cyan. 3-bio.
	Philodendron erubescens	Cyan. 3-PG.
89–293	*Erythronium citrinum*	Cyan. 3-PG. + some Cyan. di.
	Tulipa Greigii	Cyan. 3-mono.
88–292	*Maranta leuconeura*	Cyan. di.
87–286	*Tillandsia Duvaliana*	Malv. 3 : 5-di. A.

TABLE 3. ANTHOCYANINS PRESENT IN FRUITS

DICOTYLEDONS

64–229	*Jasminum humile*	Cyan. 3-mono. + some Cyan. 3-bio.
	Olea europea (Musajo 1939)	Cyan. PG.
59–212	*Fatsia japonica* (Hayasi 1939*b*)	Cyan. 3-mono.
55–194	*Citrus Aurantium* var.	Cyan. 3-mono. + some Cyan. 3-bio.
54–193	*Vitis hypoglauca* (Cornforth 1939)	Malv. 3-mono.
40–143	*Cotoneaster Francheti*	Pel. 3-mono.
	Stranvaesia undulata	Cyan. PG.
21–77	*Fuchsia*, 3 unnamed vars.	Malv. di. + Peon. di.

MONOCOTYLEDONS

89–298	*Ruscus aculeatus*	Pel. 3-mono.

Miscellaneous

22–83	*Mirabilis jalapa* (flowers)
19–59	*Phytolacca acinosa* (fruits)

These contain nitrogenous anthocyanins of the betanin type (see Ainley and Robinson 1937; Price and Robinson 1937; and Taylor 1940).

TABLE 3 (*continued*)

(27–96) *Cistus villosus*

These flowers possibly contain a new anthocyanin. The distributions of the anthocyanidin to the 'cyanidin' and 'delphinidin' reagents (Robinson and Robinson 1931) are correct for petunidin, but it gives no reaction with ferric chloride. The anthocyanin behaves as a pentose glycoside, and the colour reactions are unusually red for a petunidin derivative.

(89–293) *Lilium japonicum* (autumn colour in leaves): Cyan. 3-mono. + some Cyan. 3-bio.

FILICES: *Azolla filiculoides* (fronds). A monoglycosidic anthocyanin of the class found in certain ferns (Price, Sturgess, Robinson and Robinson 1938). A solution in 1 % HCl is yellow, and in amyl alcohol is orange pink. The anthocyanidin gives a red colour with sodium acetate and a positive reaction with ferric chloride. Its distribution to both the cyanidin and delphinidin reagents is lower than that of cyanidin.

CONIFERAE: *Picea obovata* (♂ cones): Cyan. 3-mono.

GENERAL DISCUSSION

The present facts do not materially affect the figures given previously for the frequency with which derivatives of the three anthocyanidin types occur as flower pigments. The combined results, together with those of Taylor (1940) on plants of the Galapagos Islands, are set out in table 4.

TABLE 4

	total no. examined	pelargonidin		cyanidin		delphinidin	
		no.	%	no.	%	no.	%
species	**530**	102	*19*	214	*40*	263	*50*
genera	**299**	69	*23*	156	*52*	148	*49·5*
families	**88**	33	*37·5*	64	*73*	57	*65*
orders	**54**	24	*44*	43	*80*	39	*72*

It was suggested by Lawrence, Price, Robinson and Robinson (1939) that the occurrence of pelargonidin derivatives in flowers may be associated with climatic conditions. We have made a rough classification of the natural habitats of the species (wild types, not varieties), dividing them into tropical, subtropical and temperate (including alpine regions). This classification (table 5) shows that there is a considerably higher proportion of pelargonidin anthocyanins in tropical and subtropical species than in those from temperate regions, and that delphinidin derivatives predominate in temperate and alpine plants.

TABLE 5

	anthocyanins derived from		
	tropical	subtropical	temperate
pelargonidin	28	23	21
cyanidin	28	33	112
delphinidin	16	18	194

This may be due to one of two causes:

(1) Pelargonidin derivatives may be more readily produced or maintained by the plant under tropical conditions than cyanidin or delphinidin derivatives, or

(2) Natural selection may favour the survival of red rather than blue flowered forms in the tropics.

Examination of the colour of tropical and subtropical flowers containing cyanidin or delphinidin derivatives shows that it is the second cause which is operative. It is the biological and not the chemical relationship which is the immediately effective one. Approximately 20% of these tropical flowers are very much redder than temperate flowers which contain the same anthocyanin. This generally results from the presence of deep yellow carotinoid pigments which have a strong 'background' effect; contributory causes might also be the absence of co-pigments and a low cell-sap *p*H. *Euphorbia fulgens* and *Dimorphotheca aurantiaca* are typical examples in which the 'pelargonin' colour has been achieved by such indirect means. Of the remaining 80% of tropical and subtropical species containing cyanidin or delphinidin anthocyanins, the majority are appreciably redder than temperate flowers containing the same anthocyanin, though the difference is not so marked as in such cases as *Euphorbia fulgens*. Included in this group are *Poinsettia pulcherrima* and *Camellia japonica*.

The selection of a particular anthocyanidin type, such as a pelargonidin derivative in many tropical plants, is naturally dependent on that type arising by mutation from the pre-existing form. The mutation might be from a white or yellow acyanic form to one containing the particular anthocyanin required. Or it might be from a form containing a different anthocyanin, for example from delphin to pelargonin. Many examples are known of this second type of mutation, and they have recently been discussed in a separate paper (Beale 1941). They evidently occur according to a regular system, the majority being from a wild type containing a delphinidin derivative to a mutant containing a cyanidin or pelargonidin derivative. The reverse process has been found in only one or two instances.

While it should be borne in mind that the most commonly occurring mutations are not necessarily those which are utilized in natural selection, it would be desirable to have more data on plants other than temperate and alpine.

Species of Tulipa

Tulipa is a genus whose flower colour ranges from white and yellow in the acyanic species to scarlet, red and purple. The yellow pigments which are present in the yellow-flowered species such as *T. galatica* and *T. Batalini*, and which also form backgrounds in many cyanic forms, are mainly of a carotinoid nature, though the colour due to these carotinoids may be modified to some extent by flavones or flavonols. We have observed that 1 % hydrochloric acid extracts of a number of species (e.g. *T. montana* and *T. Batalini*) deposit colourless crystals which give colour reactions resembling those of flavones or flavonols. In this respect they resemble certain *Crocus* species, extracts of whose flowers deposit crystals of kaempferol (Price, Robinson and Robinson 1938).

The petals of many of the *Tulipa* species tested are bicoloured. There is a purplish black blotch at the base of the petals while the other part, constituting the bulk of the petal area, is scarlet or red. It is the anthocyanin of this upper part of the petal which is listed in table 1. In the following species the anthocyanin responsible for the blotch at the base of the petals is a mixture of a delphinidin diglycoside and a delphinidin pentoseglycoside in various proportions:

T. linifolia
T. Maximowiczii
T. armena
T. Didieri
T. Julia
T. Stapfii
T. undulatifolia
T. praecox
T. Veneris
T. kuschkensis
T. lanata
T. sovietica
T. Eichleri
T. Fosteriana
T. Hageri
T. Whittallii
T. Ostrowskiana
T. pulchella

A similar mixture is found in the stamens of *T. praecox*. The blotch in *T. Hoogiana* contains a mixture of delphinidin and cyanidin diglycosides, whereas in *T. oculus-solis*, *T. aleppensis* and *T. Schmidtii* it consists of delphinidin and cyanidin pentoseglycosides. In the blotch of *T. Clusiana* all four anthocyanins are present, delphinidin and cyanidin diglycosides and pentoseglycosides.

As regards the principal anthocyanins of *Tulipa* listed in the table, the following comments may be made: *T. montana* is very variable in colour, so that the difference (a small admixture of cyanidin 3-pentose glycoside) between the two forms examined is not surprising. Two forms of *T. armena*, *T. Didieri*, *T. Stapfii*, *T. aleppensis*, *T. praestans* and *T. violacea*, and three forms of *T. Kaufmanniana*, were examined, and the results were in all cases concordant.

T. Fosteriana has been recorded previously, but it is included for the sake of completeness, as these data constitute the most complete survey made of any one genus. *Tulipa* comprises two morphologically distinct subgenera, the *Leiostemones* and the *Eriostemones*. All three anthocyanidin types—pelargonidin, cyanidin and delphinidin—occur in the genus, but (excluding the basal blotch) only pelargonidin and cyanidin derivatives are found in the *Leiostemones*, and only cyanidin and delphinidin derivatives in the *Eriostemones*. The occurrence of all three anthocyanidin types in the garden tulips, which belong to the *Leiostemones*, is presumably the result of artificial selection of mutants which would be eliminated in the wild.

We are indebted to the Government Grants Committee of the Royal Society for a grant in aid of this investigation, and to Mr T. H. Parsons, Curator of the Royal Botanic Gardens, Peradeniya, Ceylon, for kindly collecting and sending flowers of tropical plants.

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Morbid mitosis and the activity of inert chromosomes in *Sorghum*

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(Plates 6, 7)

Sorghum purpureo-sericeum has five pairs of active, *A*, chromosomes and a variable number of extra, *B*, chromosomes in equilibrium in the wild population (Janaki-Ammal 1940; and table 7).

The *B*-chromosomes vary in structure within and between plants owing to frequent spontaneous changes, including misdivision of the centromere. One is an iso-chromosome.

The *B*'s are *sex-limited* so far as that is possible in a plant: they are confined to the germ track owing to loss by lagging elsewhere. They are lost in the radicle before seed ripening and in the shoot tissues as they reach maturity. Only in the anthers and ovaries are they regularly maintained.

B-chromosomes pair with one another at meiosis when homologous, and the two arms of the iso-chromosome form chiasmata with one another.

Pollen grains of plus plants (with extra *B*'s) have extra divisions of the vegetative nucleus rapidly following the primary division.

The first pollen grain division is delayed by the presence of *B*-chromosomes. Its course is always normal. At the second division the *B*'s always pass to the generative pole undivided and so double its dose.

When only two generative nuclei are formed, one or both may produce sperm. Three, four or five generative nuclei, however, kill the pollen grain. The extra divisions are thus malignant.

The *B*-chromosomes as usual are heterochromatic. They have an abnormal nucleic acid cycle. Their action on the cells containing them is non-specific and cumulative, and their apparently specific effect in stimulating mitosis in the pollen grains is possibly due to these being the only cells that contain them whose mitosis and growth are normally limited.

Spontaneous structural changes in heterochromatic chromosomes are frequent at mitosis in plants and animals. Such changes could evidently establish malignant propensities in somatic cells by stimulating recurrent mitosis.

1. TWO KINDS OF CHROMOSOME

The ordinary chromosomes making up the haploid set of an individual or a species are all necessary for the regular development of every tissue and of nearly every isolated cell. Indeed, that is how we have to define a *set* of chromosomes. But there is another kind of chromosome, an extraordinary kind, which is not necessary in this absolute sense. It may be dispensed with in one of two ways, either in certain cells of the body, or in certain individuals of the population.

The same two kinds of variability affect parts as well as whole chromosomes. And for both parts and wholes the population variability exists in two states: equilibrium of different types within a group and differentiation between groups or races. A differentiation must of course always arise from an equilibrium, however short-lived. We have thus six classes, which may be represented by the following outstanding instances:

extra chromosomes	body differentiation	population equilibrium	population differentiation
wholes	*Sciara* (Metz 1938)	*Tipula* (Bauer 1931) *Metapodius* (*Y*) and *Cimex* (*X*) (Darlington 1939)	*Mesostoma* (Husted and Ruebush 1940)
		Gryllotalpa vulgaris	*Gryllotalpa vulgaris* (Steopoe 1940)
		Zea (Darlington and Upcott 1941*a*)	
parts	*Ascaris* (White 1936)	Unequal bivalent types in Orthoptera, e.g. *Gryllotalpa vulgaris*	*Drosophila pseudo-obscura* (*Y*) (Dobzhansky 1937). Other variable *Y* types (Darlington 1937*b*)

Liability to loss at mitosis is characteristic of all extra chromosomes. In body differentiation this liability becomes regulated and adapted to a physiological use which evidently preserves it. In the population-differentiated types this use has not hitherto been obvious. Now it is revealed by the equilibrium in which the extra chromosomes can exist. The equilibrium explains the origin of the differentiation and at the same time it demands that the chromosomes concerned have a use. We see therefore that the appearance of inactivity in these chromosomes was a false appearance; it was due to the absence of standards for measuring activity of the kind they show. Evidently the activity of inert chromosomes is non-specific in this sense, that the products of their activity do not interact with the products of one gene in the specifically active chromosomes rather than another.

Thus the two kinds of differentiation may be regarded as different aspects of the same fundamental problem. They help to explain one another. The importance of this conclusion has now been shown in an unexpected way: the two differentiations have been found together in the same species. In a wild diploid grass, *Sorghum purpureo-sericeum*, Janaki-Ammal (1940) has found, among a hundred plants, forty with extra chromosomes. These extras vary from one to six in number (table 7). And they are never found in the roots, but are confined to the shoot system.

In this plant species there is therefore a distinction, however rough and ready, between body and germ-track such as has been known hitherto only in animals. Three questions at once arise: First, how is this distinction brought about? Secondly, how is it maintained? and how are the chromosomes revealing it inherited? Thirdly, how do they maintain their equilibrium in the population? Dr Janaki-Ammal has kindly provided us with the plants and seeds to enable us to study these problems.*

2. ELIMINATION AT MITOSIS

The five *A* chromosomes of the standard set have submedian centromeres and are of nearly the same length. Mitosis proceeds regularly in all tissues. Five bivalents are formed at meiosis.

Irregularities were found in one plant. One or other of the chromosomes was seen to lag at anaphase in inflorescence tissue and micronuclei were formed. Some cells were seen with as many as three such nuclei, of various sizes, probably in various stages of degeneration. In cells with or without such micronuclei metaphases appeared with one chromosome missing. In some tissues nearly half the plates were deficient in this way. This indicates an even higher proportion of deficiency in the nuclei, since the deficient nuclei would be less active. One anaphase with eight chromosomes in each group was seen to be losing the ninth by lagging on the plate.

This plant was useful as showing us how chromosomes of the ordinary kind can be lost in this species. Their loss can be recognized by the formation of micronuclei. They are lost in a non-specific way, presumably owing to a mild spindle weakness. Such a weakness might be due merely to the cultural conditions. The effect distinguished between root and shoot, for the roots were normal.

Turning now to the plants with extra or *B*-chromosomes, the *plus* plants, as we shall call them, we find a different kind of irregularity. In the first place the youngest roots of the germinating seeds contain ten chromosomes. They have lost the *B*'s during seed development. In the second place the young shoots usually have the *B*-chromosomes, but loss sometimes occurs. In the growing inflorescence the *B*'s are eliminated from the parts of the flower which are not going to produce germ cells. Filaments and glumes are littered, as it were, with micronuclei. The pollen mother cells, on the other hand, contain an invariable number of *B*'s, and are free from micronuclei. The anther walls and ovaries are intermediate in the amount of loss. They have rare micronuclei.

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* For root-tips 2BE gentian violet sections (15μ) were used. All stem tissues were pre-fixed in 1:3 acetic alcohol. Young inflorescences and pollen grains were teased out and stained in aceto-carmine, pollen mother cells in acetic orcein. Pollen grains are orientated for study by sliding the cover-slip while watching the mitosis (cf. Darlington and La Cour 1941).

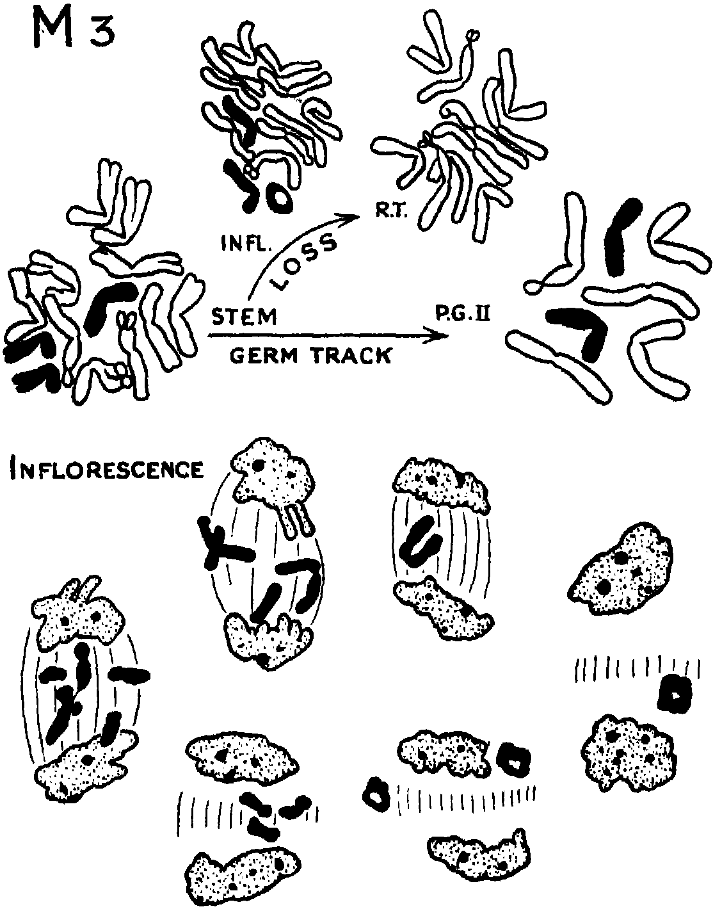

FIGURE 1. Mitosis in different tissues of M 3, showing the complement with the standard three chromosomes preserved in the young stem, being lost in the inflorescence and after their elimination in the root. Two are present at the second pollen grain division. Side views of anaphase in the flower show lagging and micronucleus formation. × 1300.

We followed this development in detail in two plus plants, which we shall refer to as LS and M3, with different numbers and sizes of *B*-chromosomes, *M*, *L* and *S* (table 1). In these plants the *B*-chromosomes are always delayed in metaphase orientation. Sometimes they altogether fail to congress on the plate, and this failure is most frequent for the *L*-chromosome, presumably owing to its exceptional size. At anaphase, in consequence, the *B*-chromosomes often lag (figures 1 and 2 and figures 13–17, plate 8) or even fail altogether in the division of the centromere. From

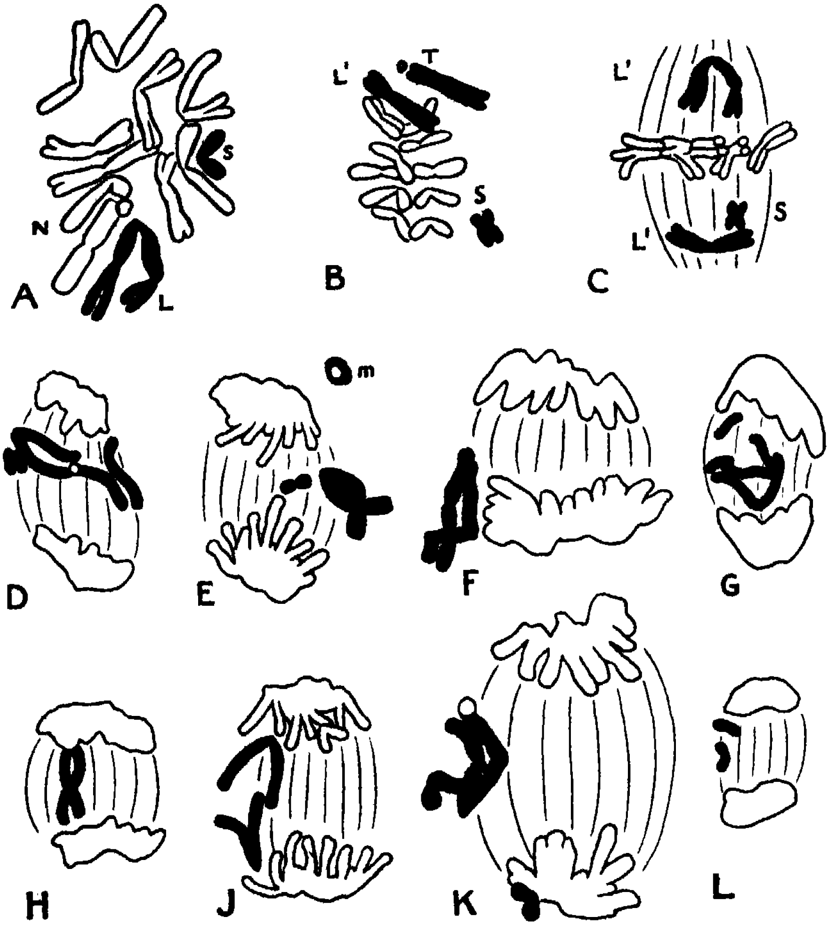

FIGURE 2. Mitosis in the young flower tissues of LS, showing lagging, loss and micronucleus (*m*) formation. *N*, nucleolar chromosome. *L'*, truncated *L*-chromosome duplicated in one cell. *T*, telocentric fragment of *L* such as is arising by misdivision in D. Different sizes of chromosomes are due to different pressures in smearing. × 1300.

this extreme case, loss and micronucleus formation is bound to result. From intermediate cases both chromatids may pass to one pole, or the centromere may misdivide, as happens with unpaired chromosomes at meiosis. Such misdivision leads to the formation of telocentric chromosomes (figure 2D) which are sometimes found at metaphase (figure 2B). Their fate we shall refer to later.

TABLE 1. SIZE AND BEHAVIOUR OF CHROMOSOMES IN *SORGHUM*

		length		estimated congression	
plant	chromosome	pollen grain μ	total in zygote μ	mitosis (glumes) %	meiosis I univalents %
Type	5A	7–9	*80*	100	—
M 3	M	6	*18*	100	*ca.* 99
LS	S	5	*23*	>95	44
	L (*iso*)	18		*ca.* 85	25

The causal sequence of loss is therefore clear. Inadequacy of the centromere, delayed congression and orientation, lagging at anaphase, micronucleus formation and degeneration. Loss occurs in one tissue and not in another, presumably for the same reason that loss occurs for *A*-chromosomes in one plant and not in another. This must be a variation during development in the activity of the spindle since such a variation can hardly be supposed in the structure or properties of the chromosome. In the track of development which leads to the germ cells the spindle is adequate even for the centromeres of the *B*-chromosomes. Elsewhere it is evidently not adequate.

3. POLLEN MOTHER CELLS

The *B*-chromosomes were present in regular numbers in all first metaphases of meiosis in the two plants.

M 3 has incomplete pairing and a low chiasma frequency. The paired configurations have a frequency of only 1·43 chiasmata (with 0·97 chiasmata terminal). The three *M*'s are evidently identical, for they form trivalents. They never associate with the *A*-chromosomes, from which, however, they are not distinguishable in size at meiosis. Table 2 accordingly gives interpretations assuming the maximum pairing of *A*'s.

LS regularly has five *A* bivalents, with 1·97 chiasmata per bivalent and complete terminalization. *L*- and *S*-chromosomes are never paired with

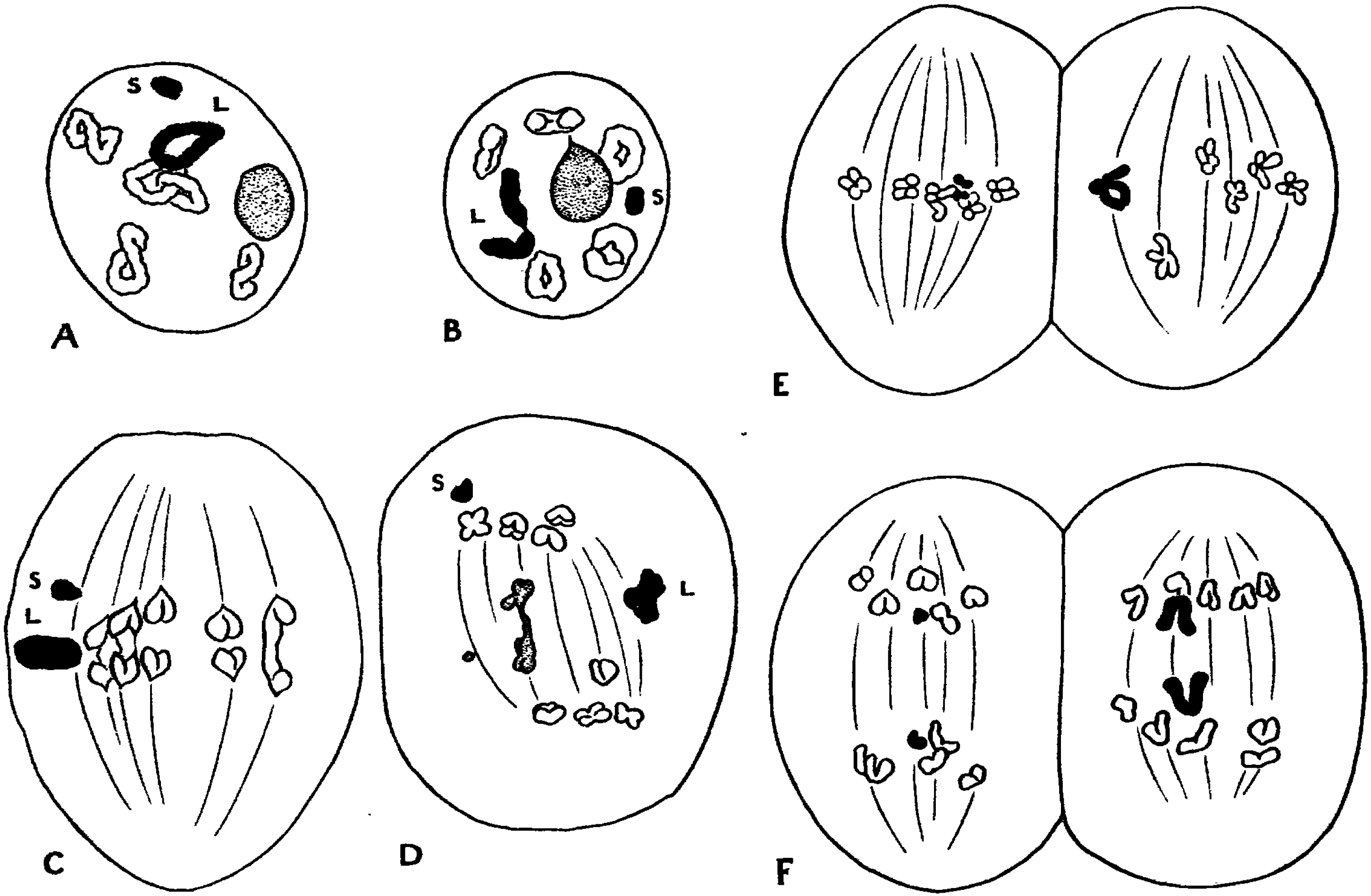

FIGURE 3. First (A–D) and second (E–F) pollen mother cell divisions in plant LS. *L* has formed one chiasma with itself in A and C, two in D where a bivalent shows inversion crossing-over. Note the absence of lagging of *L* and *S* at second anaphase. × 1300.

one another or with an *A*. Both *L* and *S*, when they come on to the plate, lie like other univalents with their bodies in the equatorial plane. The *L*-chromosome frequently forms a ring, however. Thus its two ends must be identical, like those in the secondary trisomic of *Datura* (Belling 1927). It is an iso-chromosome. The *S*-chromosome, which is too small for analysis, may have a similar stucture. Since we have seen telocentric *B*'s at mitosis, these chromosomes presumably arose on the lines described in *Fritillaria* (Darlington 1940). This origin implies a centromere of abnormal strength, other evidence of which we have already noted.

TABLE 2. CONFIGURATIONS AT FIRST METAPHASE IN M3

A's	*M*'s			
	III	II + 1	3 I	total
5^{II}	4	16	23	43
$4^{\text{II}}+2^{\text{I}}$	—	—	4	4
$3^{\text{II}}+4^{\text{I}}$	—	—	1	1
$2^{\text{II}}+6^{\text{I}}$	1	—	1	2
total	5	16	29	50

At first anaphase *L* and *S* as well as unpaired *M*'s pass to one pole without division, even in cells where metaphase congression has been perfect. At the second division they might be expected to divide, but to do so with some delay as at ordinary mitoses. It is remarkable therefore that they show no delay at all.

4. POLLEN GRAINS: FIRST DIVISION

The primary division in the pollen grains follows a regular course whether *B*-chromosomes are present or not. It also takes place at the regular time—one week after meiosis. The *B*-chromosomes themselves show some structural changes, but divide normally (figure 4). Thus in a sample of the plant M3 with eleven anaphases four had one *B* and seven had two, but in these as well as in nineteen telophases there were no laggards.

In LS different samples of pollen grains were taken and they differed in the frequency of *B*'s. This would happen if the *B*'s affected the rate of development of the grain just as ordinary chromosomes do. It is possible to measure this activity on a scale provided by the proportion of grains which have passed through their first division. The sample can then be recorded according to the length of the complement, all values above 40μ

representing B-chromosomes. In the plant LS the more B-chromosome material is present the longer is the interval required before the first division (table 3).

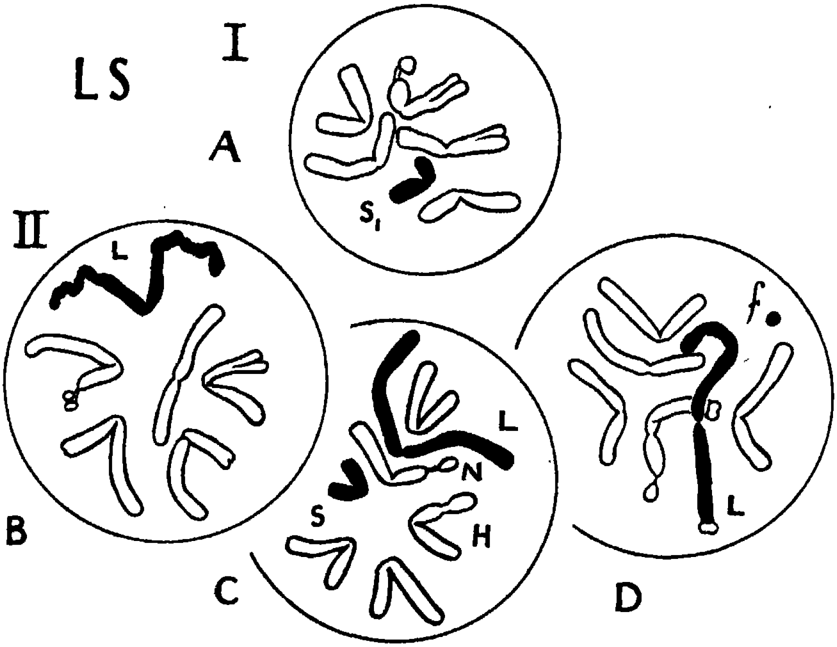

FIGURE 4. First and second (supernumerary) pollen-grain mitoses in LS. S_1, a modified S-chromosome. In C apart from that in N, the nucleolar chromosome, a second, heterochromatic, segment appears as a constriction (H). f, a fragment, probably of S. × 1300.

TABLE 3. FIRST DIVISION IN THIRTY-SIX POLLEN GRAINS OF PLANT LS

complement ...		5A	+ S	+ L	+ SL	average
$A+B$ value ...		*40*	*45*	*58*	*63*	value
proportion binucleate:*	10 %	14	3	0	0	41
	30 %	5	3	0	0	42
	50 %	1	2	1	2	52
	90 %	0	0	3	3	60·5
	total	20	8	4	5	

* A proportion of the 90 % sample had already entered into the supernumerary divisions to be described later.

When allowance is made for the inequality of our samples and of their timing, it will be seen that there is roughly a half chance of each chromosome, L or S, appearing in each pollen grain. There is therefore no evidence of loss, which we had no reason to expect, or lethality, which we might have expected. The *B*-chromosomes produce no unbalance. They are active but non-specific.

5. POLYMITOSIS

Description. In normal plants the first division of the pollen grain gives a central vegetative nucleus and a peripheral generative nucleus which, as in grasses generally, divides a week or so later to give the two male nuclei.

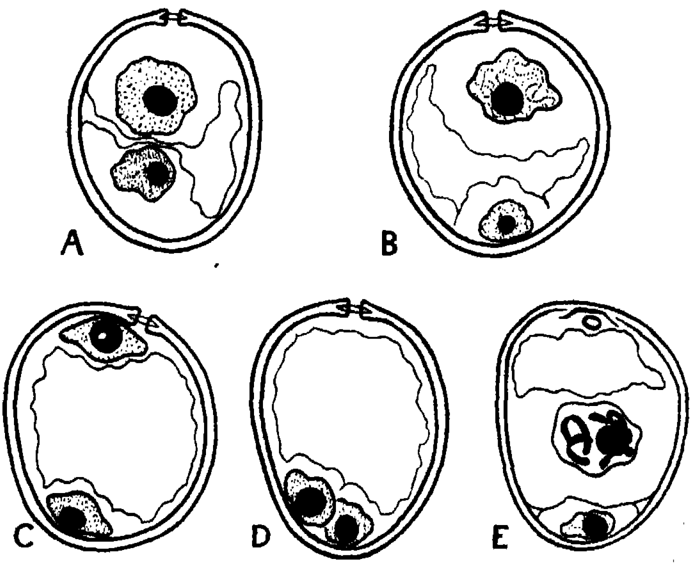

FIGURE 5. Modification of normal development of pollen grains in a diploid plant. A, subequal division of cytoplasm. B, both nuclei in a supernumerary prophase. C, probably derived from A. D, non-polarization. E, supernumerary prophase of vegetative nucleus. × 300.

Certain abnormalities occur in normal plant pollen (figure 5): (i) non-polarization, (ii) subequal division of cytoplasm, (iii) second division of vegetative nucleus.

These have been found in *Tradescantia* by Sax (1935) under abnormal conditions, and probably occur in most plants as a rarity. In all six plus plants examined, however, the last of these abnormalities, supernumerary division of the vegetative nucleus, is developed to an extent hitherto unknown. The vacuole, which normally separates the vegetative and generative nuclei, disappears and the two remain lying close together. The extra division then occurs very promptly. The rapidity of the division is indicated by the frequent persistence of relic coiling in those ends of the

L-chromosomes which have evidently been left extruding from the resting nucleus (figure 4). And then the central nucleus may repeat this process until it has piled up three, four, or even five, peripheral nuclei against the same side of the grain opposite the germ pore. These divisions follow in rapid succession, probably all within 24 hours.

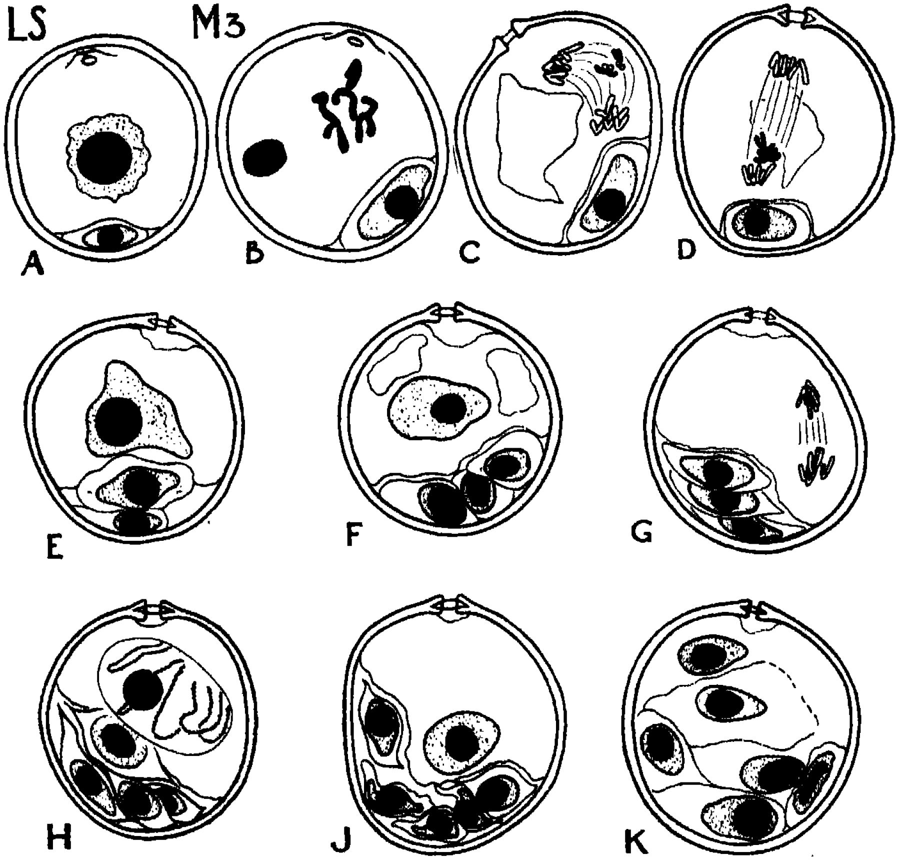

FIGURE 6. The course of supernumerary divisions all taken from one preparation of M 3 and derived from a normal binucleate cell like that of LS shown in A. B, prophase of first extra division of vegetative nucleus; nucleolus persisting. C, D, passage of *M*-chromosomes to generative pole. E, F, second and third telophase. G, fourth anaphase. H, fifth prophase. J, K, probably final resting stage, K having shifted its polarization. × 300.

At the beginning of the extra divisions the *B*-chromosomes begin to show their somatic defect. They lag on the anaphase spindle. But they are not lost. In nineteen supernumerary metaphases of M 3, one had no *M*-chromosome, eight had one and ten had two. All the *M*-chromosomes were lagging. In the early stages they were lying on the equator, later they were passing undivided towards the generative pole. Thus the vegetative nucleus does what the diploid nuclei off the germ track so regularly do—it gets rid of its extra chromosomes. But here, on this one most important occasion, the germ-track nucleus doubles its set of *B*'s.

These divisions suggest the development of the gametophyte found in the gymnosperms, except that the number of cells cut off can be greater and the cells themselves have the form, and even the function, of additional generative cells and not of additional somatic cells.

If a sample of grains is examined while this process is taking place, and while nearly all the grains are still healthy, its general character can be recorded (tables 4 and 5). We then see that in addition to a straightforward polymitosis of the vegetative nucleus a variety of other deviations can be traced. Some of them are like those found occasionally in normal plants, but they are combined with polymitosis as follows:

(i) The first division is sometimes unpolarized and transverse, giving two equal and symmetrical nuclei, as sometimes happens in normal plants.

(ii) The cell cut off with the first, second, third or fourth peripheral nucleus may be larger than usual and it may then itself divide once—as it would in a normal plant—but a week earlier (figure 7 D, E).

(iii) Secondary failure of polarization may occur, so that two central nuclei are formed after the first peripheral nucleus. These may then undergo a third mitosis with or without their previous fusion (figure 7 A, B and C).

Table 4. Vegetative (*v*) and generative (*g*) nuclei in two samples of LS pollen grains before sperm formation

		g					
	v	0	1	2	3	4	total
I	1	8^Y	19^N	4^P	—	—	*31*
	2	2^U	—	—	—	—	*2*
II	1	2^Y	49^N	13^P	3^P	1^P	*68*
	2	—	3^S	—	—	—	*3*
	total	*12*	*71*	*17*	*3*	*1*	*104*

Y, 'young' or undeveloped grain. Four in 8*Y* are degenerating, the rest are young and undeveloped. One in 2*Y* is probably diploid from restitution and has two equal nucleoli.

N, normal development.

P, straight polymitosis giving extra *g*'s.

U, unpolarized, with equal and transverse first division, giving two *v*'s.

S, secondarily unpolarized.

TABLE 5. VEGETATIVE AND GENERATIVE NUCLEI IN M3 POLLEN GRAINS BEFORE SPERM FORMATION. LATER SAMPLE THAN TABLE 4

	g					
v	0	1	2	3	4	total
1	15^{Y}	18^{N}	56^{P}	11^{P}	2^{P}	*102*
2	3^{U}	5^{S}	1^{S}	—	—	*9*
3	2^{U}	—	1^{S}	—	—	*3*
4	1^{U}	—	1^{S}	—	—	*2*
total	*21*	*23*	*59*	*11*	*2*	*116*

Y, all degenerating pollen grains. One nucleus has two small nucleoli, another was at metaphase.

N (18), one had *v* at metaphase ($n = 5 + 2M$); three had *v* at prophase. (56), one had *v* and *g* in telophase for second division, another had *v* in prophase for third division. One had *v* at pore pole, another had both *v* and *g* at pore pole. (11), one had two micronuclei from lagging *B*'s. One had *v-g* telophase. (2), one had *v-g* telophase.

S, secondary failure of polarization of *v* to give equal nuclei after budding off one *g*. One in (5) had *g* degenerated and another had one *v* at the pore pole.

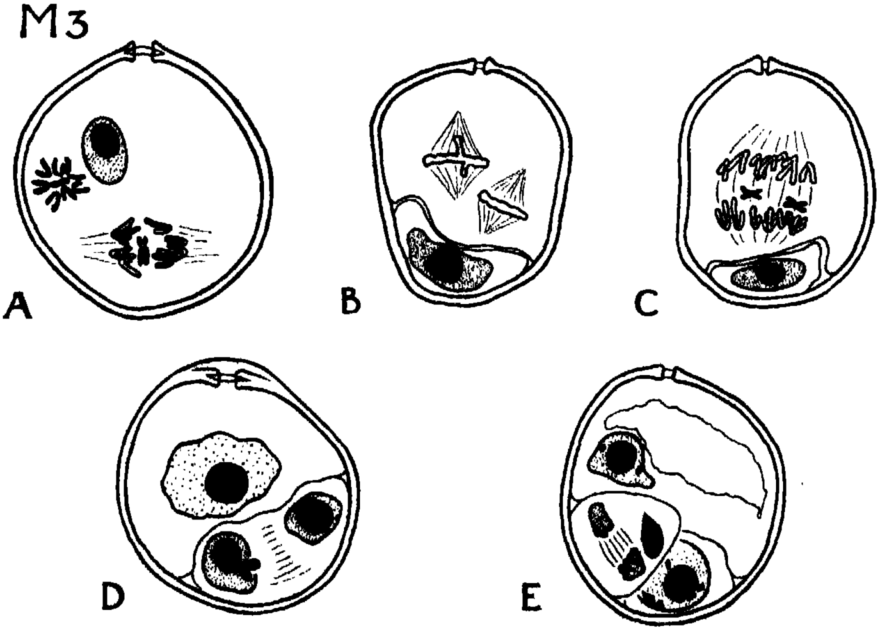

FIGURE 7. Abnormal supernumerary divisions in M3. A, third metaphase after non-polarization of second; note presence of one lagging *M*-chromosome in one division and of three in the other owing to lagging at second division. B, different view of third metaphase. C, third anaphase after fusion; lagging of two *M*'s. D, telophase of a precocious division of a generative nucleus with an extra *M*-chromosome which has lagged. E, similar precocious division of second generative nucleus with persisting nucleolus (also observed once in table 5 sample). Note, heterochromatin is abundant in the first generative nucleus but not in vegetative nucleus. × 300.

Causation. What of the *B*-chromosomes in relation to polymitosis? The extra divisions are stimulated by their presence. But does their effect persist solely from action in the maternal cell, or is it solely immediate? Or is it again both persistent and immediate? The evidence is of three kinds:

(i) *Frequency of B's at the first extra division.* In all twenty pollen grains of LS seen at the first extra division, *L* or *S*, or both were present in addition to the five *A*'s, as follows:

	LS	*L*	*S*	none
observed	12	6	2	0
expected	5	5	5	5

This selective action points to some post-meiotic effect of the *B*'s in promoting division.

(ii) *The frequencies of extra divisions.* The general samples in tables 4 and 5, in which we regard polymitosis as nearly complete, show a variation in intensity conforming with a variation in the frequency of *B*-chromosomes distributed to the pollen grains at meiosis. This also argues a post-meiotic effect.

(iii) *The behaviour of cells without B chromosomes.* At the first extra division all *B*'s, as we saw, pass to the generative pole without division. Hence the vegetative nucleus must always be destitute of *B*'s and those in the generative nucleus probably do not act on the vegetative cell. Its further rapid divisions must be due to the delayed effect of their action during the earlier period.

Thus the effect of the *B*-chromosomes on polymitosis is derived chiefly from their action between meiosis and the first mitosis of the grain affected. It is cumulative. In this respect it differs from the timed action of specific genes and resembles the effect of *Y*-chromosomes in *Drosophila* (Schultz 1941).

6. SPERM FORMATION

The ripe pollen enables us to take a more extensive view of the course of development in diploid as well as plus plants. In the normal course (as it is known in grasses generally) a week elapses between meiosis and the primary division and nearly another week between this division and the next, when, a day or two before dehiscence, the generative nucleus forms the two crescent-shaped sperms of the ripe grain. In the polymitotic grains likewise, periods of rest precede and follow the polymitosis. During the second period there is some degeneration such as always affects different kinds of grain differentially. In the ripe grains with more than one generative nucleus we then find three degrees of development: (i) no sperm, (ii) two sperm, and (iii) two pairs of sperm of unequal size. Presumably these degrees correspond to degrees of vitality, since in the ordinary grains the generative nucleus may collapse instead of forming sperm (figure 8). Degrees of vitality are also shown by the range between the long worm-shaped sperm that are commonest in the healthy grain and the short pear-shaped sperm that often appear in polymitotic grains. Vitality of sperm seems to be proportionate to length.

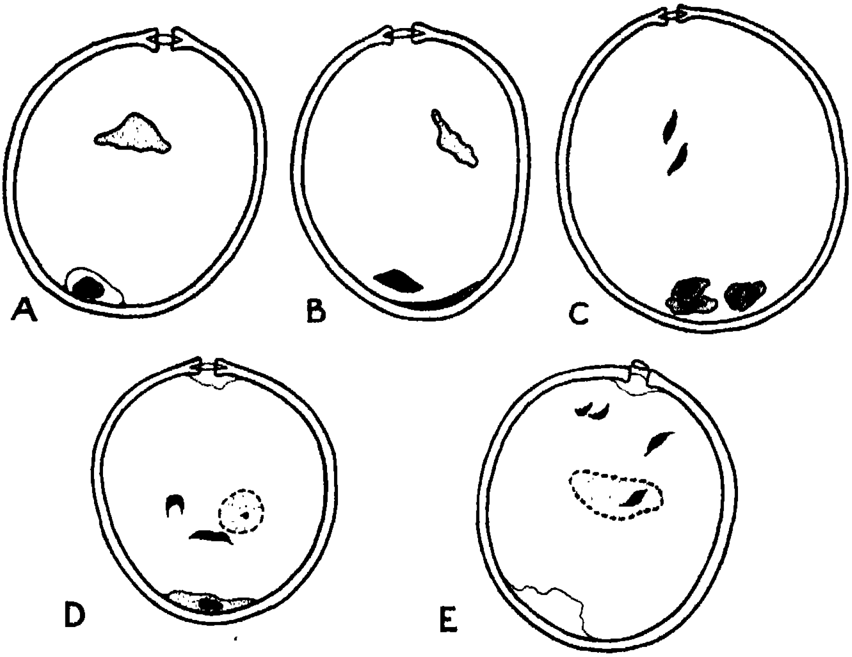

FIGURE 8. Types of ripe pollen. A–C, in plant 15 (see table 6 and figure 9; C is presumably diploid from a binucleate grain. D in plant with two *B*'s. E in plant with one *B*.

There is an enormous increase in empty grains in the plus plants with increasing number of *B*'s (Janaki-Ammal 1940). These plants, as we saw, have an increasing amount of polymitosis during middle pollen grain growth. Observation of the ripe pollen grains now enables us to extend this analysis and show the story in a new perspective (table 6 and figure 10). *First, we find that some of the diploid plants produce a small proportion of grains with two generative nuclei.* The difference of behaviour between diploid and plus plants is therefore not absolute but one of degree.

Secondly, the poorness of the sample increases with the proportion of multinucleate grains. Now none of the good grains have more than two generative nuclei or their derivative sperms, except the grain in figure 8C which is diploid. Since pollen grains with three, four or five generative nuclei are common amongst higher plus plants, it follows that the pollen which has failed is largely of the polymitotic kind, which increases in frequency with the number of *B*'s.

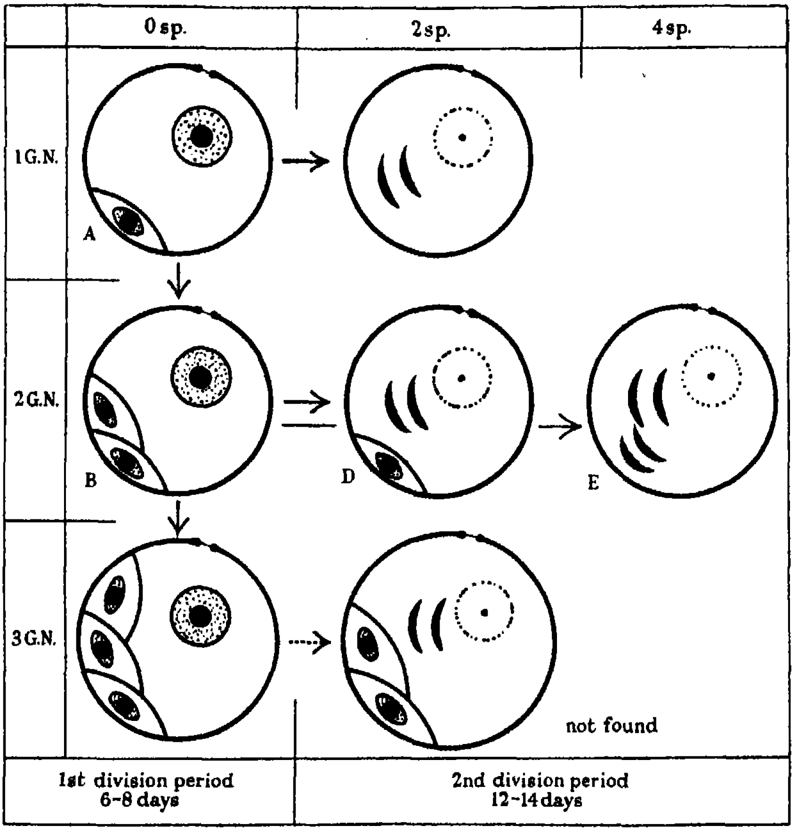

FIGURE 9. Diagram showing the presumed course of polymitotic pollen development. *The two division-periods are measured from the time of meiosis.* Sperm are probably not formed when more than two generative nuclei have been produced.

One more conclusion concerns the effect of *B*-dosage. Janaki-Ammal's observations and ours, so far as they go, show that the effect of dosage is geometrically rather than arithmetically cumulative. Two have more than twice the effect of one. In this property again, so far as we know, inert chromosomes are distinguishable from any kind of active genes.

TABLE 6. SPERM IN RIPE POLLEN GRAINS

type	plant no.	number examined	empty %	1 GN %		2 GN %			grains with sperm
				0 sp.	2 sp.	0 sp.	2 sp.	4 sp.	
$2x$	21*	*189*	**5·8**	—	94·2	—	—	—	*94·2*
	15	*165*†	**11·5**	3·0	78·2	0·6	6·1	—	*84·3*
	25	*172*	**22·7**	—	72·6	1·8	2·9	—	*75·5*
$2x+1$	22	*91*	**17·6**	—	81·3	—	1·1	—	*82·4*
	60	*86*	**29·1**	—	46·6	2·2	22·1	—	*48·7*
$2x+2$	12	*105*	**16·2**	—	23·8	9·5	47·6	2·9	*74·3*
	59	*115*	**58·3**	—	16·0	18·3	6·1	0·9	*23·0*
$2x+3$	7	*483*	**96·0**	—	1·2	0·4	2·4	—	*3·6*

* This plant produced equal numbers of pollen grains with and without starch grains. It was evidently heterozygous for a direct-acting gene controlling starch formation in the pollen, as has been found previously in *Oryza*, *Zea* and elsewhere.

† *Among these one pollen grain was diploid (figure 8C) and its nuclear differentiation* doubtful. It has been classified as 5 GN in figure 10.

Note: the sizes of the *B*-chromosomes enumerated in this table were not recorded. *L*, *M* and *S* each count one. This increases the variability within classes.

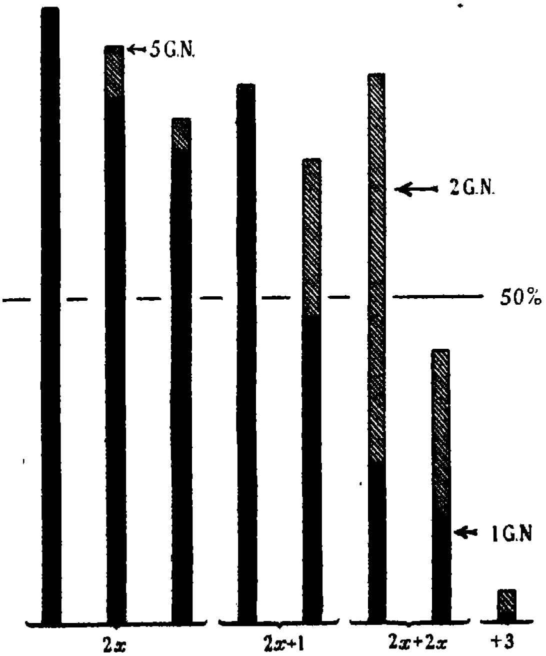

FIGURE 10. Diagrammatic representation of table 6, neglecting types of sperm formation.

7. HETEROCHROMATIN AND NUCLEOLI

The genetic 'inertness' of the *B*-chromosomes, their frequent structural changes, their new homologies all suggest an analogy with heterochromatic chromosomes elsewhere and make it necessary to examine their nucleic acid cycle. Their absence from root tips and the impracticability of freezing make the enquiry more difficult than usual.

The standard complement contains a number of small heterochromatic segments which are stained in resting nuclei of various tissues. Evidence of one of these has been seen at metaphase (figure 4C). The *B*-chromosomes, occurring only in shoot tissues, seem to be heterochromatic, but variable in behaviour. Possibly the degree of their condensation varies with the activity of the cell and the size of the nucleus. The micronuclei to be sure are strongly condensed, but a degenerating nucleus might well be so, whatever its chromosome constitution. More satisfactory evidence is obtained from three special sources. (i) In the extreme case of a plant with five *B*'s, five large deeply condensed bodies, in addition to the small ones, could usually be recognized in anther wall tissue (figure 11). (ii) Pachytene in plus plants shows large blocks of heterochromatic material resembling the *B*-chromosomes of *Zea Mays*. (iii) There is a difference between the first generative nucleus and the vegetative nucleus after its second division in figure 7E. The one which we expect to have the *B*-chromosomes shows several larger bodies of heterochromatin, the other only two smaller ones.

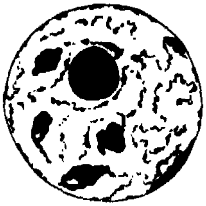

FIGURE 11. Resting stage in anther wall nucleus of plant with five *B*-chromosomes, showing their heterochromatic character. × 1300.

Thus the exact plotting of the position of heterochromatin is not possible, but its concentration in the *B*-chromosomes is clear.

One of the most difficult but promising peculiarities of the polymitotic pollen grains is the variation in the sizes of nucleoli. The nucleolar organizer presumably lies in the secondary constriction seen in one of the *A* chromosomes at mitosis (figures 1–4). The nucleoli are as a rule large, and this is particularly striking in the smaller generative nuclei. Further, we see that when the generative nuclei with large nucleoli divide, the nucleolus is too large or the cell too small for rapid dissolution in the normal way. The nucleolus persists to anaphase and is rejected on the side of the spindle. This kind of abnormality is common in unbalanced pollen grains and has even been seen in root-tip mitoses of plus plants in *Zea* (Darlington 1937) and *Tulipa galatica* (Upcott, unpublished).

Why should nucleoli be upset in a plant with an excess of *B*-chromosomes? The *B*-chromosomes are heterochromatic. Caspersson and Schultz (1940) have shown in *Drosophila* that the nucleic acid/protein ratio of nucleoli depends on the amount of heterochromatin in the nucleus, although heterochromatin is concerned with the metabolism of *thymo*-nucleic acid and nucleoli contain *ribose*-nucleic acid. It seems therefore, from their observations and ours alike, that the carbohydrate components of the two nucleic acids, the one in the nucleoli, the other in the chromosomes, are interchangeable within the nucleus.

Another clue to this relationship which should be borne in mind is the absence of large and visible heterochromatic segments in those species of the Paridae with nucleolar organizers (Darlington 1941).

8. CHROMOSOME DIMINUTION AND CUMULATION

We have seen that the *B*-chromosomes in *Sorghum* must have defective centromeres which make it possible for them to be eliminated at any stage of development at which the mitotic spindles are also defective. Now defective centromeres are well known in *B*-chromosomes elsewhere (e.g. in *Zea Mays* and *Tulipa galatica*) and are even perhaps characteristic of them. Yet nowhere else in plants is there any evidence of a differentiation in their behaviour as between tissues. It therefore follows that this differentiation must have arisen in *Sorghum* as a special combination of changes such as can only be adaptive. This is all the clearer for the obvious disadvantages which the *B*-chromosomes bring to the pollen that bears them. Polymitosis is a form of cell suicide that must require powerful compensating advantages for it to survive.

The doubling of the *B*-chromosomes in the second generative nucleus will, if this nucleus functions, have an important bearing on this question. For if the average content of *B*'s is increased mechanically in every generation an opposite physiological selection must reduce their frequency to an equal extent.

Comparison of previous accounts shows that doubling happens in the same way in plus plants of *Secale*, although at the first pollen grain division; a second division not having been found in rye.

Hasegawa (1934) found that *Secale* with two extra chromosomes ($14+2B$) formed 98 % pollen grains with $7+B$ chromosomes. These by primary division gave the following separations of *B*'s to the vegetative and generative nuclei in twenty-one pollen grains:

entirely to generative nuclei	equal division	lost	entirely to vegetative nuclei
13	2	5	1

In other words, the majority of male gametes from $14+2B$ plants had $7+2B$ chromosomes. As in *Sorghum* the segregation of the undivided *B* in some cells goes with its loss in others and must be attributed to a defective centromere.

From these observations, so remarkably similar to our own, we should expect an excess of progeny with even numbers of *B*-chromosomes. This happens in *Sorghum*, as Janaki-Ammal found, and it happens in *Secale* even more strikingly, as the data brought together in table 7 now show for it affects the progeny on the female side as well as on the male side.

The mechanical *cumulation* of *B*-chromosomes in the generative nuclei of the pollen grain, i.e. in the male germ line, of *Secale* and *Sorghum* has far-reaching implications. It means that their equilibrium in the population may depend on the opposite conditions to those in *Zea Mays.*

TABLE 7. INCREASE OF *B*-CHROMOSOMES AND EXCESS OF EVEN NUMBERS IN PROGENIES OF *SECALE*, ACCORDING TO LEWITSKY, MELNIKOV AND TITOVA (1932) AND GOTOH (1932), SHOWING ANALOGY WITH JANAKI-AMMAL'S NATURAL POPULATION IN *SORGHUM*

		progeny							total		
author	parents	0	*B*	2*B*	3*B*	4*B*	5*B*	6*B*	plants	*B*'s	*M*
G	**O × BB**	5	2	8	—	—	—	—	*15*	18	**1·2**
G	**BB × O**	2	—	3	—	—	—	—	*5*	6	**1·2**
L	**O × BB**	2	2	29	1	—	—	—	*34*	63	**1·9**
L	**BB × O**	4	—	10	—	—	—	—	*14*	20	**1·4**
L	**BB × BB**	—	—	4	1	10	—	—	*15*	51	**3·4**
L	**4B × 4B**	1	—	1	—	4	—	2	*8*	30	**3·8**
J	Nat. Pop.	60	12	20	5	1	1	1	*100*	82	**0·8**

In *Zea* there is a mechanical loss of *B*-chromosomes in the germ track and this implies a compensating physiological advantage in the presence of *B*'s for the species, or rather an optimum above the observed mean. In *Secale* and *Sorghum* on the other hand the mechanical gain implies a physiological disadvantage for the species, an optimum below the observed mean. Such a contrast is not so surprising as it may seem. It merely means that (i) the heterochromatin supply of the standard complement is below the optimum requirement in all three species, (ii) that it is inaccurately adjusted to this optimum by means of *B*-chromosomes and (iii) that it is under-adjusted in *Zea*, over-adjusted in *Secale* and *Sorghum*. This error of adjustment is in all three cases due to their mechanical errors.

9. MALIGNANT MITOSIS

What we have just described as polymitosis differs from the condition so described by Beadle (1931) in a fundamental respect. In *Zea* multiple division is imposed by the cell on the nucleus before the necessary nuclear change of chromosome division has taken place. It is the spindle that takes the lead. In *Sorghum*, on the other hand, multiple division is imposed by the nucleus on the cell. The chromosomes always divide perfectly, and such abnormality as takes place is due to under-development of the spindle. We may suppose indeed that the spindle is barely able to keep up the pace of division set by the nucleus. Again, as we might expect from its extra-nuclear origin, the defect in *Zea* is determined by a single recessive gene operating maternally and not directly in the individual pollen grains. In *Sorghum* the defect is determined by a heterochromatin excess which probably continues to operate in the individual haploid cells as well as by delayed maternal effect.

Why does polymitosis occur in plus plants of *Sorghum* at this particular stage of development? For specific genes we cannot say what determines specific timing in their actions, beyond generally stating that their actions are reactions with a system which is co-ordinated in time. In this heterochromatic effect, however, we are dealing with a continuation of a process which is normally interrupted at this stage.

The grains are sealed up in their walls a few days after meiosis. When this cell wall is not formed, as we now find in the Orchidaceae (Barber 1941), the co-ordination in timing of the whole loculus is maintained. When the wall is formed, each grain becomes a physiological unit liable to its individual errors. At the same moment its growth is stopped, but mitosis continues, without cell-wall formation, in the presence of an excess of nucleic acid.

From this point of view the nuclear polymitosis in the pollen grain is analogous to malignant growth of a mature animal cell. It differs in that each malignant cell is already imprisoned in a thick cell wall. Each pollen grain is an *encapsulated tumour*. Though the limited resources of the pollen grain lead to suicide, with unlimited resources proliferation would presumably follow. It may be (on a neo-Weismannian view) that the loss of *B* chromosomes in plus plants of *Sorghum* before the maturity of somatic tissues avoids this proliferative excess. Or, again, it may be that where every cell is equally a plus cell the competition of equals gives regulated growth.

Although few enough observations have been made capable of testing the relation of heterochromatin, that is of nucleic acid metabolism, to cell division, we may recall:

(i) the great variability in timing and internal co-ordination of pollen grain divisions in triploids (Upcott 1939);

(ii) the occasional occurrence of supernumerary divisions in the vegetative nucleus in triploid *Tradescantia* (Sax 1937) and triploid *Lilium* (cf. Coulter and Chamberlain 1915);

(iii) the precocious division of a generative nucleus in triploid *Tulipa praecox* (Upcott 1939); and

(iv) the occurrence of extra divisions of the vegetative nucleus in *Amaryllis Belladonna* with two extra and probably 'inert' chromosomes (Upcott 1939; cf. Sato 1938).

(v) the entry of the pollen tetrad nuclei into an abortive prophase immediately after meiosis in the old triploid garden tulip Zomerschoon (Upcott unpub.).

All these cases are plus plants or triploids. Plus plants will always have a nucleic acid excess. The triploids, if their heterochromatin is unequally distributed amongst their chromosomes, will likewise have it unequally distributed amongst their pollen grains, sometimes with excess and sometimes deficiency of nucleic acid. This is borne out by the frequency of undissolved nucleoli in their pollen grains. In every instance we therefore have reason to associate nuclear polymitosis with nucleic acid irregularity.

The opposite side of the picture is perhaps shown by Dobzhansky's inter-racial hybrids in *Drosophila* (1934). $B \times A$ are deficient in the inert Y-chromosome as compared with the B parent. Meiosis comes too early in the development of the follicle, and its precocity is only partly made good by a succession of 'supernumerary' mitoses.

We saw earlier the important distinction that can be drawn between nuclear and cytoplasmic polymitosis. Now malignant cell division must always be derived from nuclear rather than from cytoplasmic disturbance. For if division of the cell is to be repeated it is essential that the division of the chromosomes shall be correct. Nucleic acid supply must therefore be a crucial variable in the causation of cancer.

How then could abnormal heterochromatin content arise in animal tissue to account for malignant growth? The answer is indicated by the high frequency with which spontaneous structural change occurs in heterochromatic segments of chromosomes (Darlington and La Cour 1940; Barber and Callan 1941). This spontaneous change could appear as the mutation to which cancerous growth has sometimes been ascribed (Lockhart-Mummery 1941). Mutations determining malignant mitosis have been produced by X-ray irradiation in *Antirrhinum* and perpetuated for several generations (Stein 1935). The frequency of spontaneous changes can be modified and conditioned by both genotype and environment, with complex results (Darlington and Upcott 1941*b*), indeed with what have been reasonably described as mixed results (Strong 1941). For a test of the consequence of such changes on mitosis, no tissue will be found to equal in experimental convenience the pollen grains of plants with varying numbers of inert chromosomes.

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DESCRIPTION OF PLATES

PLATE 6. Mitosis in the plant LS

FIGURE 12. Root-tip metaphase. Note two satellite chromosomes. Flemming-gentian violet section (× 3000).

FIGURE 13. Anaphase showing *L*-chromosome forming separate spindle on the equator (× 2000).

FIGURE 14. Metaphase as in figure 2K and resting cell with micronucleus (× 1800).

FIGURE 15. *L*-chromosome lying off metaphase plate (× 2000).

FIGURE 16. Anaphase as in figure 2J (× 2000).

FIGURE 17. Late anaphase. *L*-chromosome lagging, *S*-chromosome at one pole. Micronucleus lying apart (× 2000).

FIGURES 18, 19. Pollen grain metaphases in LS. Figure 18, first division as in figure 4A (× 2000); figure 19, same with normal *S*-chromosome.

FIGURE 20. Second pollen grain metaphase in LS with *L* and *S* in addition to five *A*-chromosomes (× 2000).

PLATE 7. Meiosis in pollen mother cells of LS (× 1500)

FIGURE 21. Diakinesis; five *A* bivalents, *L* and *S* univalent. *L* as a ring.

FIGURE 22. Five *A* bivalents, *S* univalent, *L* broken into two telocentrics as in figure 2D.

FIGURE 23. Group of metaphases with *L* and *S* unpaired, and one anaphase with *S* lagging.

FIGURES 24–26. First anaphases. Figure 24, *S* lagging, *L* at one pole. Figure 25, both lagging. Figure 26, *L* almost broken by stretching of spindle.

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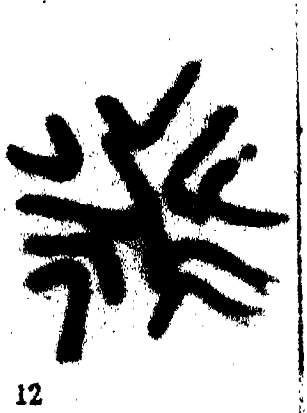
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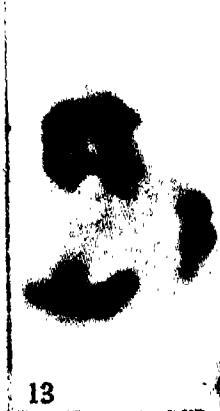
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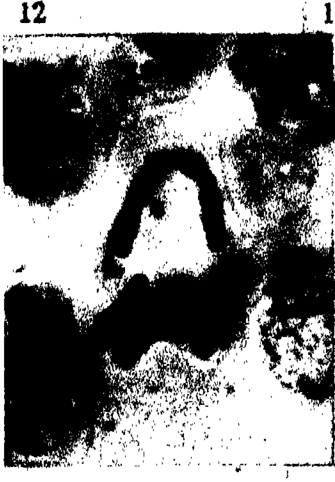

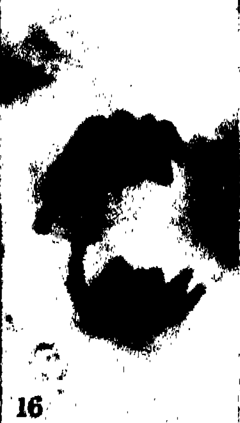
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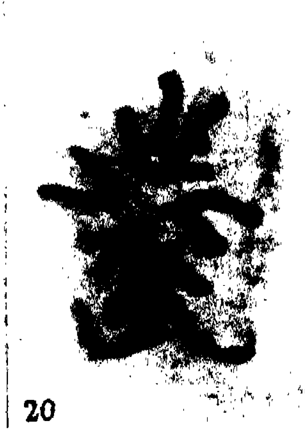
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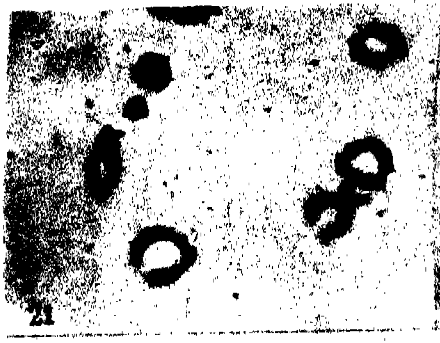
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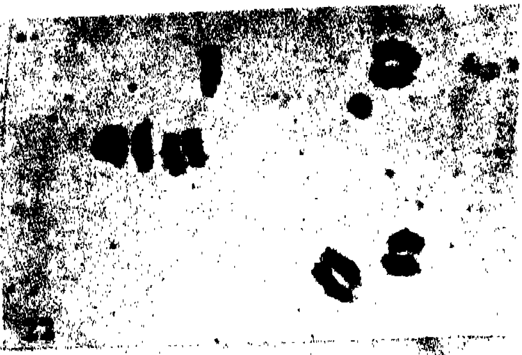
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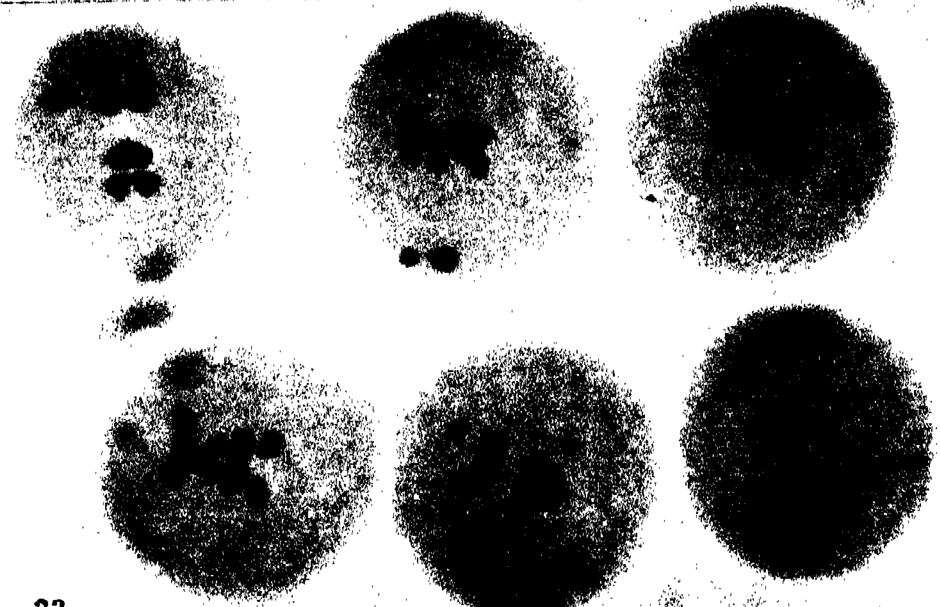
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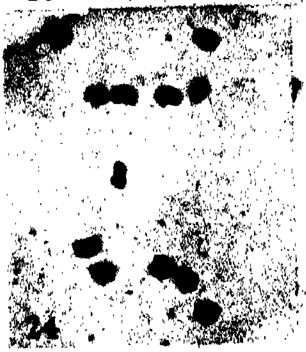
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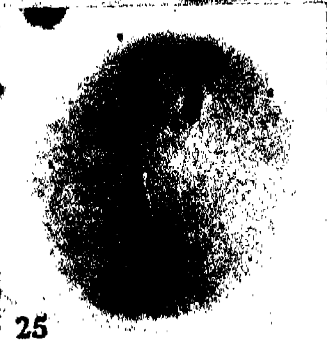
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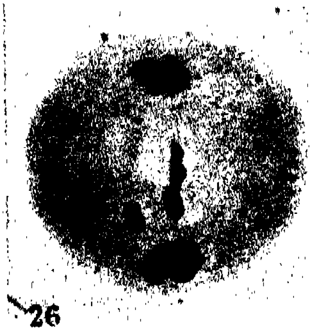
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The relation between nutrition and exhibition of the gene Antennaless (*Drosophila melanogaster*)

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The gene *Antennaless* located on the second chromosome of *Drosophila melanogaster* prevents formation of antennae under appropriate conditions of culture. In normal cultures phenotypic indices (§ 1) of pure lines are at first high, then decline to a minimum about the fourth day, and subsequently rise to near the initial level.

This *emergence effect* is not due to selective mortality of genotypes distinguished by modifiers of Antennaless. It is due to external changes in normal culture media.

As indicated by the temperature-effective period of the normal fully fed larva, the time of action of the gene is between the third and fourth days of larval life.

Within a small range examined (5° C), temperature changes have a striking effect on exhibition, when the medium is sterile. On normal media, changing behaviour of yeast populations masks this effect.

An apparent association between changing pH of a normal medium and changing frequency of exhibition in a normal culture is brought about by a complex series of interacting agencies.

It is possible to correlate successive changes of exhibition frequency in a normal culture with: (*a*) gross changes due to the activities of the larvae; (*b*) biochemical changes in the yeast diet arising from differential fertility or mortality of yeast strains and from the direct action of changing conditions upon the metabolism of the microflora.

Yeasts produce X substances which encourage, and Y substances which inhibit, exhibition of the gene Antennaless. The relative proportions of X and Y substances vary in accordance with: (*a*) yeast strains used in the same environment; (*b*) different external conditions to which the same strain responds.

A provisional hypothesis suggested by data derived both from study of gross changes in a normal culture and from analysis of yeast fractions is that: (*a*) transition from the descending to the ascending phase of exhibition coincides with change from aerobic to anaerobic respiration of the yeasts; (*b*) this change of yeast metabolism affects the relative concentrations of specific nutritional products which influence exhibition.

Among yeast constituents normally required by *Drosophila* one fraction, which is adsorbed by charcoal, lowers the frequency of exhibition. Vitamin B_1 has a similar effect, and is partly responsible for the observed inhibitory effects of certain yeast fractions.

1. INTRODUCTION

The topic indicated by the title of this communication signifies that it deals with phenotypic variation in a *pure* line. It is based on a mutant type not hitherto described. We must first explain the circumstances of its origin and evidence for recognizing a unique relation between: (*a*) the visible effect (exhibition) of the presence of the gene, (*b*) the changing environment of a normal fruitfly culture.

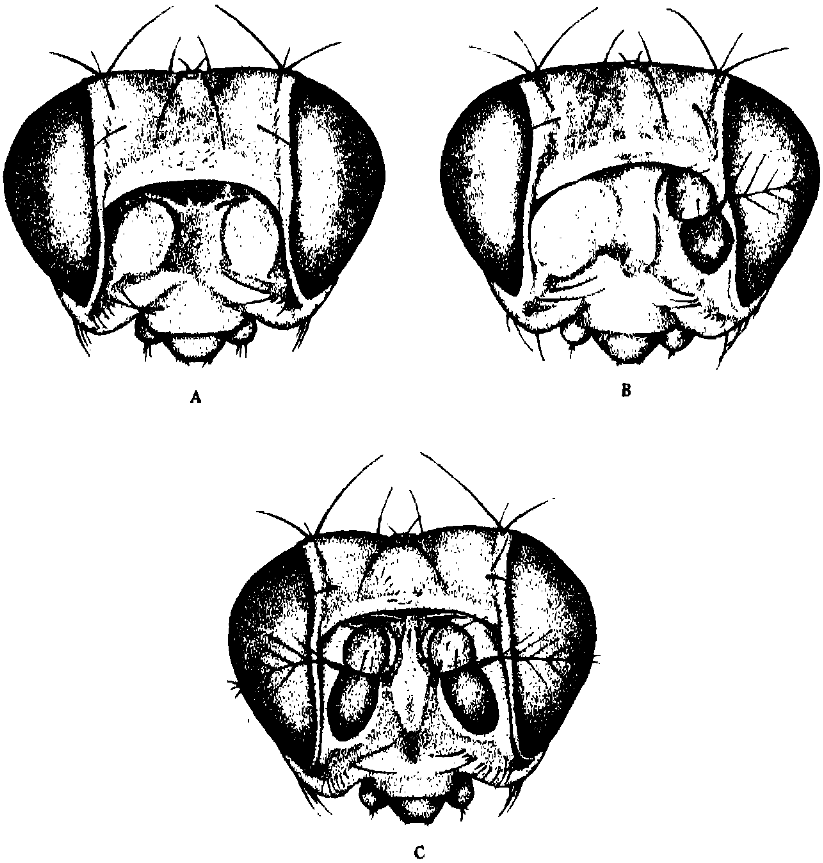

FIGURE 1. Phenotypes of the mutant Antennaless. A = bilateral Antennaless (Ant.), B = unilateral Antennaless (½ Ant.), C = wild type (W.T.).

While examining mutants which one of us (C. G.) obtained by inbreeding, Frances Gordon discovered *Antennaless* (figure 1). Mutants so named turned up in very small numbers in the later stages of a Rough-eyed culture. A single cross between a virgin Antennaless female and an Antennaless male established a homozygous stock for this gene. Localization technique referred the latter to the second chromosome. Experiments directed to more precise definition of the Antennaless locus were handicapped by what we shall call an 'emergence effect'. High exhibition occurred among early flies and low exhibition among later ones. This recalls what happens in cultures of *Abnormal Abdomen* (Morgan) and *Eyeless*; but the decline does not continue indefinitely. After a preliminary fall the percentage of mutant type goes up.

In what we shall later prove to be homozygous stocks, flies may be bilaterally antennaless or wild type, and between the two extremes we can sort out various antennal abnormalities including unilateral suppression. No single clear-cut criterion of exhibition can therefore cláim precedence. Accordingly, we shall explore variability of exhibition with three indices (α, β, γ) which give weight to different criteria. In the succeeding formulae: a = number of bilaterally antennaless flies; b = number of flies not included under (a) or (c); c = number of wild-type flies:

$$\alpha = \frac{a+b}{a+b+c}, \quad \gamma = \frac{a}{a+b+c}, \quad \beta = \tfrac{1}{2}(\alpha+\gamma).$$

In conformity with usual practice the upper limit of all three formulae is unity, and the lower is zero. Figure 2 shows the typical exhibition-time graph, based on formulae α and β.

Most of the first-day flies are bilaterally antennaless. The proportion declines gradually up to the fourth or fifth day. Most flies are then wild type or unilaterally antennaless. The proportion of bilaterally antennaless flies rises to the original level about the ninth day. The form of the curve is therefore consistent with changing environmental conditions operating first to lower then to raise gene exhibition.

Figure 2 refers to a stock produced for high exhibition by selection and inbreeding through fifty generations. During the first ten generations we selected flies for high exhibition. Thereafter the stock continued to propagate by random brother-sister mating. Over the whole of this period the exhibition-time graph preserved its characteristic shape. Of itself, this clearly points to one conclusion. Unless regular changes in the culture medium are correlated with differential viability of genotypes, the existence of wild-type flies in the culture cannot be due to heterozygosity with respect to modifying genes. We can exclude this alternative.

When inbreeding does not reduce diversity within pure lines, there is a conclusive way of recognizing differences due to environment. We can compare the progeny of parents with or without a mutant character, or of parents which exhibit different grades of it. In so far as the range of phenotypic variation is not affected by the choice of parents, it has its basis in external agencies. Table 1 shows the results of such a test after fifty successive generations of inbreeding. The figures stand for values of the index α, as defined above, for offspring of different pairs of sibs whose phenotype (W.T. = wild type, Ant. = antennaless) is stated at the side of the two rows. They refer to observations covering 2 days after the beginning of emergence. The table shows that phenotypically wild-type and antennaless parents produce the same proportion of antennaless offspring.

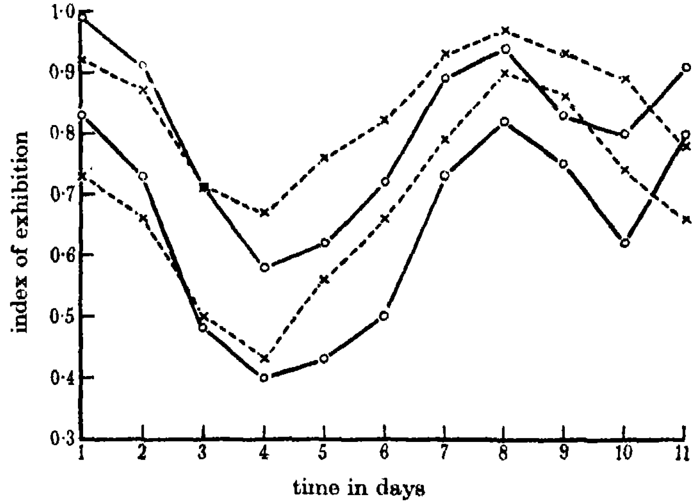

FIGURE 2. Normal exhibition-time graph. × = males: ○ = females. Upper pair of curves α; lower pair β.

TABLE 1. OFFSPRING OF PHENOTYPIC MATINGS FROM PURE LINES (50 ± GENERATION)

								mean
Ant. × Ant.	0·85	0·86	0·94	0·93	0·95	0·99	0·95	0·91
W.T. × W.T.	0·83	0·91	0·97	0·89	0·96	0·97	0·93	0·93

We have thus a clear-cut relation between exhibition and changing conditions of environment in the normal culture. Previous investigations furnish us with little guidance for further elucidation of this phenomenon. Earlier workers who studied nature-nurture interaction were preoccupied with the controversy concerning transmission of so-called acquired characters. When controversy subsided the relation of genes to chromosomes overshadowed other topics. So there have been few detailed studies on how external agencies limit gene exhibition. The most notable concern mutants of *Drosophila melanogaster*, e.g. the work of Morgan (1929) on *Eyeless*, Morgan (1915), Schäffer (1935) and Braun (1938) on two different genes responsible for *abdominal* deformities, and Krafka (1920) and others on *Bar eye*. Apart from the last, which deals with graded exhibition in relation to a specified agency (*temperature*), these studies point to one predominant limiting agency, the moisture content, of the normal medium. Morgan suggested that the influence of moisture may be indirect, i.e. through the yeasts which are the actual food of *Drosophila* larvae. Other workers have described a 'starvation' effect (Beadle, Tatum and Clancy 1938; Child 1939), i.e. mutant strains may produce wild-type flies under conditions of generalized food shortage. Beadle and Law (1938) have shown that bacterial action can bring about production of *vermilion* substance. With this exception no one has yet induced mutant strains to revert to wild type by addition of normal food constituents, nor has anyone found that dietetic treatment can produce forms with mutant traits.

One thing emerges clearly from such fragmentary observations. In study of how exhibition varies with external circumstances or with changes in the normal medium, current methods of culture provide us with no means of distinguishing between two issues. One is the direct effect of the *physical* environment upon developing eggs or larvae. The other is its indirect effects due to presence of a *growing* yeast population. A culture is a highly complex ecological system. External changes may produce consequences by selective mortality either of flies or of micro-flora. They may also promote accumulation of varying proportions of excretory or other byproducts by their action on the metabolism of both.

So much was clear to one of us (C. G.) when the other author (J. H. S.) independently surmounted technical difficulties of culturing *Drosophila* on media containing no live yeasts. All experiments recorded subsequently are the result of co-operative work to which *each author* contributed his own technical experience. Since we started with a blank sheet, we have had to explore many blind alleys. For the benefit of investigators who might otherwise waste time and effort, we shall place on record protocols of experiments with negative results along with others which justify positive conclusions. A sufficient reason for doing so is that our first failures focus attention on neglected ecological aspects of gene exhibition.

2. MATERIALS AND METHODS

Details of the composition of the culture media are as follows:

(*a*) *Maize meal-molasses-agar medium*, subsequently referred to as the 'normal' medium. (*a*) 4000 c.c. water, 60 g. agar; (*b*) 380 c.c. water, 355 g. crude molasses; (*c*) 1200 c.c. water, 720 g. maize meal. Where specified the 'anti-mould' Nipagin (methyl-parahydroxy-benzoate) was added to the medium. Solution *b* is added after slowly dissolving the agar. Mixture *c* is then added and the whole mass slowly boiled until the consistency is judged to be such that the maize meal will be distributed evenly throughout the mixture when cool, when about 50 c.c. is poured into each culturebottle.

(*b*) *Sterile media.* One of us (J. H. S.) has tested various types of synthetic or semi-synthetic sterile media proposed in studies on the nutritional requirements of *Drosophila* (van't Hoog 1936; Lafon 1937; Tatum 1939). None of them was satisfactory. All prolonged the larval period excessively, and the resulting flies were smaller than normal. So media having killed yeast as larval food replaced them. Sterile media containing 4% yeast incorporated in an agar gel gave good results; but the consistency of the medium varied considerably after autoclaving, and even more so when the pH was altered by adding various buffers. This change altered the rate of larval development. To avoid this we tried out a medium without agar. Yeast and sawdust in the ratio 1 to 2 (± 1) by weight are mixed thoroughly, and 1 g. placed in each of the 6 × 1 in. experimental tubes. To this 3·5 c.c. of Pearl's S. 101 salt solution is added.* After soaking for about 1 hr. the tubes are stoppered and autoclaved. We were able to get comparatively consistent results with this medium.

(*c*) *The culture set-up.* (i) *Normal medium.* Three pairs of flies are mated in a vial containing the normal medium. These are then shaken into the yeasted culture bottles kept in an incubator at 25° C. The flies begin to emerge after the ninth day. This day's yield is termed the first day's count. Since circumstances affecting exhibition are numerous, a separate control accompanied every experiment.

(ii) *Sterile medium.* Pearl's S. 101 medium is allowed to set in the lids of dipping jars, each containing about 100 flies. The latter lay eggs in the Pearl medium. The lids can be changed at convenient intervals. The eggs are then sterilized with White's fluid (White 1931) while still in the lids, washed in sterile distilled water and covered with sterile Petri dishes. Immediately after hatching the larvae are transferred to the sterile medium with a platinum spoon.

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* Pearl and Penniman (1926), made up thus: sodium potassium tartrate, 8 g.; tartaric acid, 5 g.; ammonium sulphate, 2 g.; magnesium sulphate, 0·5 g.; potassium dihydrogen phosphate, 0·65 g.; calcium chloride, 0·25 g.; nipagin, 1·0 g.; sugar, 30 g.; water to 1 l.

From these particulars the reader will see that there is one important difference between the set-up of experiments involving use of normal and sterile media. In normal cultures parent flies continue to lay eggs during the whole period before emergence of the youngest progeny. With the exception of those recorded in § 7, all observations on sterile media were made on larvae of the same age.

3. Effect of hydrogen-ion concentration

The first clue we followed was suggested by correspondence between the exhibition-time graph of a normal culture (figure 2) and the changing hydrogen-ion concentration of the normal medium (figure 3), as described by Bridges and Darby (1933). In these experiments we made direct measurements of the changing pH of a normal medium with a Beckman Glass Electrode Potentiometer. To get a quick reading the medium should

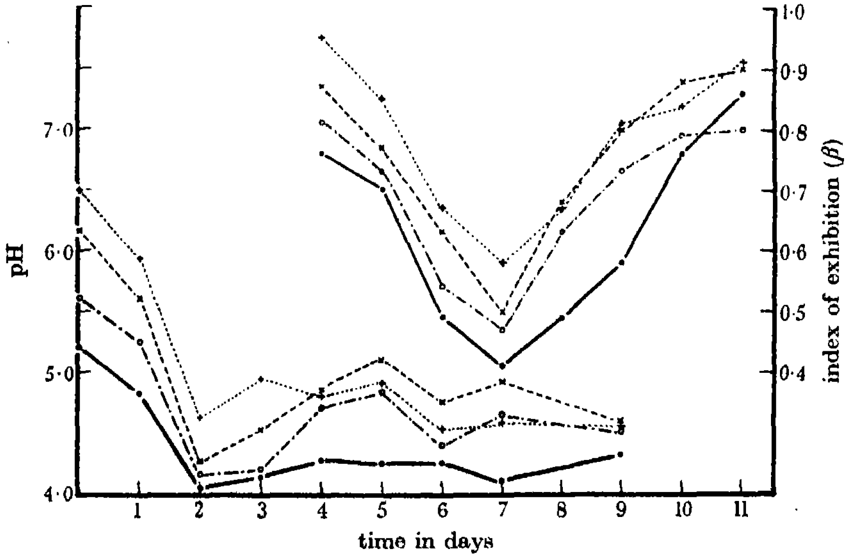

FIGURE 3. Exhibition and pH changes on normal medium buffered with Na_2HPO_4; no nipagin. Lower group of curves = pH; upper group = exhibition (displaced 4 days). ● control: ○ 0·2 g. Na_2HPO_4 per 100 c.c. medium; × 0·6 g. Na_2HPO_4; + 1·2 g. Na_2HPO_4.

be mashed, and the electrodes pressed well into the pulp. The pH is not uniform at different levels. Larvae and yeasts are mainly confined to the surface of the medium in earlier stages, and to relatively small depth later. So only the top layer of the medium was removed for examination. We made all measurements at constant temperature in a thermostatically controlled room.

To interpret this correspondence correctly it is necessary to take into account a conclusion established at a later stage (§ 5*b*). The gene Antennaless acts between the third and fourth day of development. This means that the pH measurement relevant to first-day emergences is that of the fourth day in the life of the culture, that pH measurements relevant to second-day emergences correspond to that of the fifth day, and so forth. We have designed figure 3 on this assumption. There is a caveat to this. Owing to variable duration of larval life, correspondence stated above is not absolute. Figure 3 shows the relationship between exhibition and pH. Broadly speaking, the exhibition index increases with increasing pH between the limits from 4·0 to 5·0. The correspondence reappeared in a repeat experiment with a medium containing *nipagin*. To our surprise we could not reproduce it when we cultured flies on sterile media buffered at corresponding pH levels. Table 2 summarizes an experiment of this class.

TABLE 2. EFFECT OF pH ON EXHIBITION ON STERILE MEDIA (McILVAINE'S BUFFER)

pH	3·8	4·2	4·5	4·6	4·7	5·1	5·3	6·2
α	0·87 ± 0·03	0·93 ± 0·03	0·85 ± 0·04	0·87 ± 0·03	0·93 ± 0·02	0·94 ± 0·02	0·88 ± 0·03	0·97 ± 0·01

This makes it clear that pH differences as such do not directly affect the exhibition of the gene Antennaless. Any effect due to pH variation must arise from (*a*) changing the metabolism of the yeast, (*b*) encouraging the survival of particular strains of yeast. To our sterile cultures containing an acetate buffer we therefore added live brewer's yeast. In such cultures the exhibition indices do not increase with increasing pH. At about 5·0 the reverse is true. This suggests that the second of the alternatives stated last is correct. To exclude the contribution of effects due to specific ions we therefore carried out a series of experiments in which we used H_2PO_4, $NaPO_4$, Na_2HPO_4, and Na_3PO_4 in equivalent concentrations of the PO_4 ion added to a live yeast (normal) medium. No orderly relation between level of exhibition and initial pH was detectable (figure 4). From this we may conclude that *varying* hydrogen-ion concentration as such does not influence the yeast diet of the flies. It is an incidental consequence of other and more fundamental processes in a medium containing a growing yeast population. Taxner (1935), Hjört-Hansen (1939) and others have shown that variation in pH has an effect on the quantity of yeast produced, but there is disagreement about whether it increases or decreases with increasing pH. Such disagreement may well be due to the use of different species of yeasts. In the light of experiments recorded in § 8 it is likely that pH increase may have different effects at different pH levels. Until we know more about the role of pH in yeast metabolism, further investigation concerning pH changes is not likely to clarify our knowledge of agencies affecting exhibition of Antennaless.

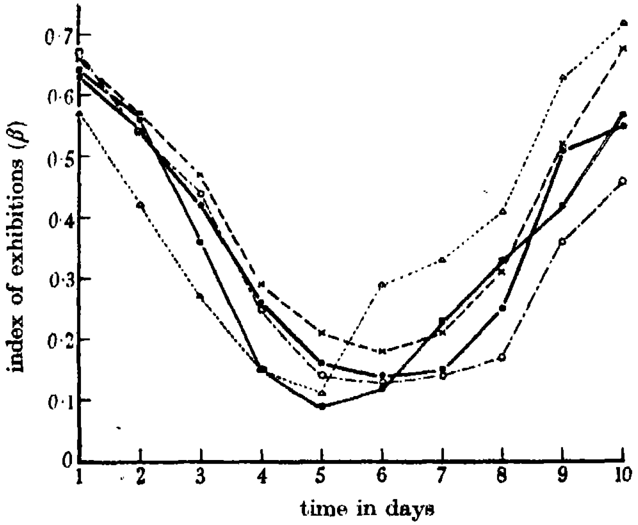

Figure 4. Effect of buffer on exhibition, phosphate kept constant.
● control; ○ Na_3PO_4; × Na_2HPO_4; ■ NaH_2PO_4; △ H_3PO_4.

4. Effects of temperature

The influence of temperature on exhibition also depends on whether the medium contains live yeasts. Except when otherwise stated we have carried out all experiments here recorded in an incubator kept at 25° C. We have also performed experiments on normal and sterile media at a lower temperature. A striking rise of exhibition indices accompanies a drop of 5° C, if the medium is sterile. The same drop produces no detectable shift on a medium containing live yeasts. Table 3 shows the contrast. The figures for the normal medium refer to the entire period of emergence.

TABLE 3

temp. °C	sterile (brewer's yeast)		sterile (baker's yeast)		normal	
	α	β	α	β	α	β
25	0·85	0·63	0·55	0·34	0·41	0·29
20	1·00	0·99	0·85	0·63	0·41	0·29

Needless to say, the period of emergence is more protracted at the lower temperature. So the exhibition-time graphs of cultures kept on a normal medium cannot coincide. If we make the total period of emergence our unit of time, they tally closely (figure 5). The conclusions to which this contrast leads us are: (*a*) temperature has a direct effect on exhibition; (*b*) it changes the proportion of yeast metabolism by-products by selection or direct action; (*c*) its effect on the proportion of these by-products is antagonistic to its direct action on the development of the antennal bud. Since many workers (*vide* Goldschmidt 1938) have studied the effects of temperature without recognizing the possible interplay of direct effects on larval development and indirect consequences of changing the microflora of a culture, all conclusions suggested by previous work, other than experiments of the 'shock' type, require re-examination in the light of the antagonism established by our own experiments.

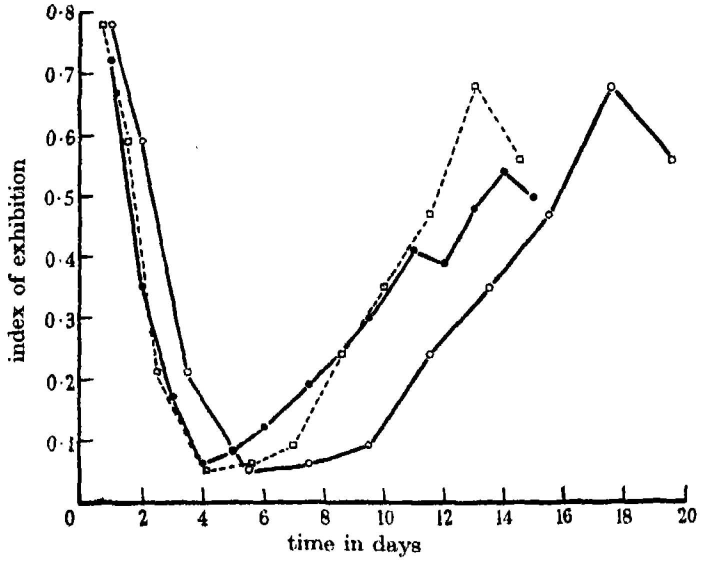

FIGURE 5. Effect of temperature on daily exhibition. ● at 25° C; ○ at 21° C.; □ at 21° C; ordinates adjusted so that 1·3 days ≡ 1 day at 25° C.

5. THE CRITICAL PHASE OF DEVELOPMENT

Our observations on pH changes and associated variability of exhibition in cultures containing live yeasts have emphasized the need for information about the time at which external circumstances can affect the action of the gene in the developmental process. With more precise information of this kind at our disposal we can also get additional evidence to exclude the possibility that differential mortality of genotypes plays any part in the phenotypic variability of our cultures.

(*a*) *Mean length of larval life.* To correlate changes of exhibition with changing ecological and physical features of a normal culture we must be able to antedate the birth of each fly from the time of emergence. In a culture on which flies are laying eggs continuously, individuals may develop early because they start earlier as eggs, or because they develop more quickly. To know the limits of birth-date error it is necessary to explore the limits within which length of larval life also varies. We have done so in the following way. Eighty newly hatched larvae were placed daily in bottles over a period of 10 days. The first set received only Antennaless larvae on the first day and only Ebony larvae on the succeeding days. The second set received Antennaless larvae on the second day and Ebony larvae on the first, third, and subsequent days. And so on. In each set of bottles for every day in the age of the culture there was thus a group of Antennaless larvae isolated in a population of Ebony larvae readily identifiable for counting on emergence. Table 4 records raw data of experiments done in this way. Analysis of the daily totals shows that the mean length of larval life increases steadily. For example, larvae put in on the fourth day (bottles 14–17) take roughly 1½ days longer to complete development than those put in on the first day. Although we cannot define the precise environmental conditions of the larval period of an individual fly one thing is clear. The exhibition-time graph of experiments carried out in this way is essentially like that of the usual type of culture containing growing yeasts.

Summarized in another way (table 5) data from the foregoing experiments confirm the conclusion that differential mortality of phenotypes is not responsible for the emergence effect.

(*b*) *Stage of larval life when changed conditions operate.* It is difficult to delimit precisely at what period in the history of a culture larvae of any particular fly will be present in the medium. So it is not easy to design experiments to establish the critical period of larval life in relation to environmental changes. Various workers (Plunkett 1932; Driver 1931;

TABLE 4. ANTENNALESS EBONY ALTERNATION EXPERIMENT 1

The numbers in brackets designate successive days' seedings—see table 5.

	bottles 1–4				bottles 5–10				bottles 11–13				bottles 14–17				daily totals				
day	ant.	½ ant.	wt.		ant.	½ ant.	wt.		ant.	½ ant.	wt.		ant.	½ ant.	wt.		ant.	½ ant.	wt.	α	β
1	174	19	2	1	—	—	—		—	—	—		—	—	—		174	19	2	0·99	0·92
2	5	—	—		64	73	11	2	—	—	—		—	—	—		69	73	11	0·93	0·69
3	1	—	4		53	92	30		3	18	18	3	—	—	—		57	110	52	0·76	0·52
4	—	—	—		1	3	1		7	21	12		—	—	—		8	24	13	0·71	0·44
5	—	—	—		—	—	—		22	32	10		14	10	17	4	36	42	27	0·74	0·54
6	10	25	50	5	—	—	—		3	2	2		67	40	23		80	67	75	0·67	0·51
7	41	63	40		5	3	2	6	1	1	—		28	9	4		75	76	46	0·77	0·57
8	12	10	5		143	66	18		—	—	—		3	1	—		158	77	23	0·91	0·76
9	—	—	1		86	21	9		1	12	11	7	—	—	—		87	33	21	0·83	0·74
10	—	—	—		17	6	1		74	5	—		—	—	—		91	11	1	0·99	0·93
11	—	—	—		2	—	—		27	—	1		—	—	—		29	0	1	0·97	0·97

Child 1934; Goldschmidt 1938; etc.) have already explored the action of temperature on exhibition of mutant genes at different stages of larval life in normal culture media. They have assumed that the *temperature-effective period* is equivalent to the time of action of the gene. The following figures illustrate the effect of temperature on the exhibition of the gene Antennaless in the *sterile* medium containing *brewer's* yeast.

TABLE 5. ANTENNALESS EBONY ALTERNATION EXPERIMENT 2

Numbers of survivors and indices of groups of larvae seeded on successive days (see table 4)

day of seeding	1	2	3	4	5	6	7
α	0·97	0·87	0·72	0·80	0·63	0·92	0·91
no. of surviving per bottle	53	55	51	54	64	63	43

TABLE 6. VARIATION OF EXHIBITION WITH TEMPERATURE

temp. °C	α	γ
25	0·85 ± 0·04	0·41 ± 0·05
20	1·00 ± 0·01	0·97 ± 0·01

Thus a temperature drop of 5° C increases exhibition considerably. With a sterile medium containing baker's yeast (Perolin) the result is similar. We kept flies of the following experiment (table 7) for varying periods at 25° C and then transferred to 21° C. Each reading refers to sixty larvae 3 hr. old when put in the incubator at 25° C.

TABLE 7. TEMPERATURE-EFFECTIVE PERIOD

time kept at 25° C. days	0	1	2	3	4	6	10
α	0·85 ± 0·05	0·94 ± 0·04	0·89 ± 0·03	0·84 ± 0·03	0·75 ± 0·06	0·78 ± 0·06	0·55 ± 0·07
γ	0·41 ± 0·06	0·38 ± 0·07	0·49 ± 0·07	0·42 ± 0·06	0·20 ± 0·06	0·14 ± 0·05	0·12 ± 0·04

The data indicate: (*a*) no consistent difference of exhibition when flies are kept at 25° C for 0, 1, 2, 3 days before transfer to 21° C; (*b*) a difference between the foregoing and flies kept for 4 and 6 days at 25° C before transfer to 21° C; (c) a difference between the latter and flies kept at the lower temperature for the complete period. Differences between individual cultures are not statistically significant; but the difference between the groups referred to in (*a*) and in (*b*) is clear-cut. Thus the *temperature-effective period* is between the *third and fourth* days of larval life. This coincides with the segmentation of the antennal bud (Balkashina 1929).

(*c*) *Sex differences.* Figure 2 shows that males and females are subject to similar changes; but on any particular day the level of exhibition is different for males and females. The sequence of these differences, confirmed on many occasions, is as follows. On the first and second day the exhibition of females always exceeds that of males. On the fifth and subsequent days exhibition of males always exceeds that of females. Differences on the third and fourth days may well be due to sampling errors. Previously recorded information with reference to the temperature-effective period suggests a plausible explanation of significant sex differences. All *Drosophila* workers agree: (*a*) that females predominate in the first day's emergence, (*b*) the sex ratio becomes 1 : 1 about the fourth day. Males and females differ with respect to relative length of pre-adult life. Apart from Bonnier's work (1926) dealing with early emergence, there is nothing to show whether this difference occurs in egg, larva or pupa. Since environmental conditions affecting the exhibition of Antennaless operate in larval life, the supposition that the larval life of females is shorter than that of males is enough to explain the sex differences in figure 2.

6. NON-SPECIFIC NUTRITIONAL LEVELS IN RELATION TO EXHIBITION

In a normal culture on which flies are laying eggs continuously the density of larval population is not constant, and available food supply may well depend upon the density level. In so far as exhibition in a normal culture depends on ecological relations of a changing larval and a changing yeast population, we have therefore to explore two possibilities: (*a*) what effects crowding may have upon exhibition frequency; (*b*) within what limits generalized food shortage may also effect the frequency of exhibition.

Since weight is a good index of access to food, we have recorded mean weights of successive batches of emerging flies from three different culture media on which parent females deposited eggs continuously. Table 8 gives data of one series. Two similar experiments confirmed the conclusion to which it points. On each of these three media containing different food constituents a continuous fall of the weights of emerging flies accompanies an initial fall and a subsequent rise of exhibition indices.

Clearly greater population density might influence development in more ways than one. For instance, accumulation of larval excretory products in the medium might directly influence the metabolism of the larvae themselves, or act indirectly by modifying metabolic processes of the microflora. Another series of experiments has shown that crowding does influence the incidence of exhibition at mean body weight levels comparable to those recorded in table 8. In the experiments summarized in table 9 we did not allow the flies to deposit eggs. We transferred 3-hour-old larvae to the same amount of two culture media. One was a molasses-maizemeal-agar gel containing a standard initial ration of live brewer's yeast. The other was the usual sterile medium containing brewer's yeast. In these experiments we varied the number of larvae and recorded the mean weight of flies at emergence.

TABLE 8. WEIGHT CHANGES IN RELATION TO EXHIBITION IN NORMAL CULTURES

day of emergence	1	2	3	4	5	6	7	8	9	10	11	12	13
mean wt. per fly in mg.	0·92	0·97	0·85	0·93	0·79	0·89	0·76	0·86	0·89	0·82	0·80	0·76	0·64
β	0·80	0·59	0·38	0·22	0·14	0·10	0·10	0·14	0·16	0·17	0·21	0·24	0·23

TABLE 9

initial density of larvae per vial	20	40	60	80	100	120	140	160
live-yeast medium:								
% survival	83	70	84	81	81			
mean wt. in mg. per fly	0·90	0·71	0·80	0·80	0·72			
α	0·56 ± 0·06	0·43 ± 0·08	0·28 ± 0·07	0·34 ± 0·05	0·10 ± 0·03			
killed-yeast medium:								
% survival	63	43	75	81	55	57	80	64
mean wt. in mg. per fly	1·12	1·15	0·86	0·70	0·69	0·59	0·33	
α	0·92 ± 0·05	0·92 ± 0·05	0·96 ± 0·03	0·82 ± 0·05	0·62 ± 0·07	0·54 ± 0·06	0·71 ± 0·04	0·32 ± 0·05

The data shown in table 9 are representative of many experiments, and may be summarized as follows. When there are live yeasts in the medium exhibition declines steeply with increasing density of larval population. In the range of density to which this statement applies the mean body weight of the flies lies within the limits recorded in table 8. On the sterile medium incidence of exhibition did not significantly drop before the density of the larval population reached a critical level near the upper limit recorded for the live-yeast medium. Thereafter it oscillated about a lower level. Throughout the whole range of experiments on the sterile medium the body weight of emerging flies declined steadily to a figure much lower than that of flies kept on a live-yeast medium.

To put this contrast in its proper perspective we have to take into account a fundamental difference between conditions of life on the two media. The food potential of a sterile medium falls throughout the life of the culture. That of the live-yeast medium is continuously replenished. In so far as it is legitimate to regard body weight as an index of generalized food shortage, we are therefore forced to two conclusions: (*a*) shortage of food in general *can* depress the incidence of exhibition; (*b*) food shortage does *not* account for the initial fall of the exhibition-time graph when flies lay eggs continuously on a medium containing growing yeasts. Provisionally we can also state a more positive conclusion. Increasing density of larval population may well account for this initial fall. To explain how the incidence of exhibition varies in relation to larval population density in an ecological system containing live yeasts and live larvae, we have now to examine two possibilities. One is what effects accumulating larval products may have on the larvae themselves. The other is how the complex interaction of a larval and a yeast population may affect accumulation of specific products of yeast metabolism.

7. ACCUMULATION OF LARVAL METABOLITES

An experiment designed to explore any effects due to accumulation of larval excretory products in normal media also yielded independent confirmation for the conclusion that variable exhibition has its basis in external agencies. To settle the issue we have to eliminate effects due to changes in the composition and condition of the microflora. We therefore allowed sterilized flies from the completely sterile medium to lay their eggs on a sterilized normal culture medium to which we added excess of dried baker's yeast (Perolin), before putting it in the autoclave to kill the micro-organisms. With the exception of those of the first day, emerging flies gave the same daily index of exhibition with only minor fluctuations. Though statistically significant, the initial deviation might be accounted for by slight fluctuations of temperature in the incubator. The experiment summarized in table 10 showed that variable exhibition in a normal culture is *not* due to accumulation of larval metabolites, and hence, in all probability, to some environmental agency directly or indirectly associated with yeast metabolism.

TABLE 10. EXHIBITION OF FLIES ON NORMAL MEDIUM *STERILIZED*

day of emergence	1	2	3	4	5	6	7
α	0·71 ± 0·03	0·52 ± 0·05	0·54 ± 0·05	0·57 ± 0·05	0·55 ± 0·05	0·50 ± 0·04	0·57 ± 0·05
γ	0·23 ± 0·03	0·13 ± 0·03	0·19 ± 0·04	0·14 + 0·03	0·16 ± 0·03	0·16 ± 0·03	0·18 ± 0·04
mean body wt. mg.	0·94	0·96	0·89	0·90	0·86	0·84	0·79

8. EFFECTS DUE TO THE BRAND OF YEAST

All the evidence set forth so far points to the necessity of getting more information about changes which occur in the yeast populations of a normal culture, or, more especially, about how growing yeast populations produce specific food constituents of the larval diet. Till recently, available literature has given us little information about how growing yeasts may produce specific nutritional constituents (Guillermond 1920; Stephenson 1930; Hartelius 1934; etc.). Williams (1941) has now shown that certain 'nutrilites' ('Bios' and other vitamin-like substances) are relevant to yeast metabolism. Though we know little about their occurrence in media we have used, they may well be significant in this context. In a search for new clues which might throw more light on the contribution of the microflora, we first examined effects due to use of different brands of yeast in making up both normal and sterile media.

We have deliberately used commercial unstandardized brands of yeast in the hope that their taxonomic heterogeneity might be helpful. Hind (1940) describes their manufacture; but not the 'stock'. Even such classical stock as 'Carlsberg' is known to be a mixture of species. So our own brands are presumably heterogeneous. We have used four in the present work: (*a*) dried brewer's yeast; (*b*) *Perolin*, a dried baker's yeast; (*c*) *Chieftain*, a wet baker's yeast prepared by the same manufacturers; (*d*) *Hutchison's* wet baker's yeast. Table 11 shows the influence of three of them on exhibition when used as the food source on sterile media.

On media containing brewer's yeast the incidence of exhibition was higher than on media containing both the brands of baker's yeast specified in table 11, as also the other (Hutchison's) mentioned above. The χ^2 test shows that variation of data derived from different cultures containing one and the same medium is due to chance. Differences associated with different brands of yeast are clearly significant. The figures for body weight show that they are not due to differences of total food content. We have independently confirmed this conclusion by growing larvae on sterile mixtures of brewer's and 'Perolin' yeast with excess of food (table 12). The shift of exhibition indices tallies roughly with the quantity of either type of yeast present. This clearly points to the conclusion that differences recorded in table 11 are due to variation of *specific food constituents* in different brands of yeast.

TABLE 11. EXHIBITION AND BRAND OF YEAST ON STERILE MEDIUM

type of yeast	number of cultures	significance test p	α	γ	wt. in mg./fly	
					males	females
baker's yeast, 'Chieftain' (wet) 6 %	5	0·50	0·26 ± 0·03	0·03 ± 0·01	1·02	1·56
baker's yeast, 'Perolin' (dry) 4 %	5	>0·05, <0·10	0·84 ± 0·02	0·37 ± 0·03	1·34	1·48
brewer's yeast, (dry) 4 %	5	>0·10, <0·20	1·00 ± 0·01	0·86 ± 0·02	1·05	1·50

TABLE 12. MIXTURE OF BAKER'S AND BREWER'S YEASTS

	100 % brewer's	75 % brewer's 25 % baker's	50 % brewer's 50 % baker's	25 % brewer's 75 % baker's	100 % baker's
α	0·92 ± 0·03	0·84 ± 0·04	0·84 ± 0·04	0·72 ± 0·04	0·73 ± 0·04
γ	0·63 ± 0·04	0·39 ± 0·04	0·41 ± 0·04	0·31 ± 0·04	0·28 ± 0·04

We have also explored the influence of different brands of yeast on exhibition in the *normal medium*. To establish a significant difference of this kind we have to exclude: (*a*) differences arising from the initial density of the seeded population of the yeasts themselves; (*b*) differential growth of other constituents of the microflora. We therefore standardized the quantity of seeded yeast by a visual test based on relative turbidity. To cultures containing different brands of yeast were added equal quantities of the anti-mould *nipagin*, which does not prevent yeast growth in concentrations which destroy other fungi, viz. 0·08 g. per 100 c.c. of water in the medium. Figure 6 shows the results of such experiments. Contrary to expectation exhibition is higher in a normal culture containing live baker's (Hutchison's) than in one which contains live brewer's yeast. The difference exists even in cultures containing equivalent quantities of nipagin.

We have thus to explore two issues: (*a*) what constituents of *dead* yeasts are relevant to the fact that substitution of baker's for brewer's yeast *lowers* exhibition in a sterile medium; (*b*) what characteristics of the growth or metabolism of *live* yeasts are relevant to the fact that substitution of baker's for brewer's yeast *favours* exhibition of the gene Antennaless.

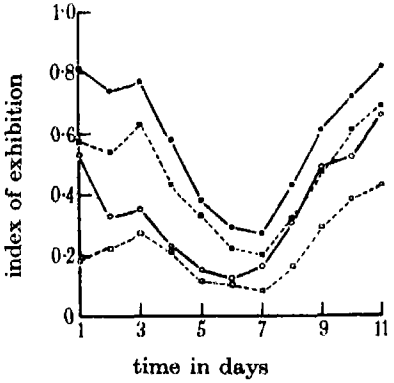

FIGURE 6. Yeast brand effects on exhibition. ● Hutchison's yeast; ○ Hutchison's yeast with nipagin; ■ brewer's yeast; □ brewer's yeast with nipagin.

9. THE ROLE OF VARIOUS CONSTITUENTS OF THE MEDIUM

(*a*) *Standardization.* Even a sterile medium is not a homogeneous or constant environment. It undergoes changes involving *inter alia* amount of free moisture and accumulation of excretory products. Larval development follows a course which depends on a sequence of such changes, and it is not practicable to reproduce them. This imposes one limitation on the technique of adding putatively active substances to a sterile medium. We can make a satisfactory comparison of sterile media containing different ingredients, only if we add the latter at the beginning of the experiment and then leave the culture to run its normal course. One set of experiments which illustrates the need for this precaution is the following. We added yeast extracts at different times to one batch of cultures and a corresponding quantity of extract-free fluid to a control set. We then applied the χ^2 test to exhibition indices of the control group. The probability that variations associated with introduction of these small quantities of fluid were due to chance alone was of the order of 0·01. Though data of table 11 show that exhibition indices of different cultures are statistically homogeneous under standardized initial conditions, there was the same variation in the control group when we took every precaution to ensure sterility of fluid introduced. In all other experiments involving sterile media we have added nothing after seeding larvae.

(*b*) *The peptone effect.* Variation of the water content of a sterile sawdust medium containing either dead baker's or dead brewer's yeast, does not significantly affect exhibition of the gene Antennaless. The same is true of the results of adding various amounts of cholesterol (0·0025–0·011 %), of sucrose (1–8 %), of metallic salts in the proportions prescribed by Pearl's S. 101 formula, or of peptone added to a medium containing dead baker's yeast. Contrariwise, the addition of peptone to our sawdust sterile media containing dead brewer's yeast significantly shifted exhibition indices. Table 13 shows the influence of peptone on exhibition in cultures kept on the two types of sterile media.

TABLE 13. EFFECT OF PEPTONE

medium	I. brewer's yeast				II. Perolin yeast				
% peptone	0	1·25	3·0	5·5	0	0·93	2·13	3·70	4·60
α	0·84 ± 0·03	0·89 ± 0·03	0·90 ± 0·03	0·95 ± 0·03	0·51 ± 0·04	0·43 ± 0·05	0·47 + 0·04	0·52 ± 0·04	0·50 ± 0·04
γ	0·44 ± 0·04	0·50 ± 0·05	0·62 ± 0·05	0·74 ± 0·04	0·07 ± 0·02	0·09 ± 0·03	0·10 ± 0·03	0·08 ± 0·02	0·12 ± 0·03

TABLE 14

% peptone	0	1	3
α	0·79 ± 0·04	0·84 ± 0·03	0·92 ± 0·03
γ	0·30 ± 0·04	0·45 ± 0·04	0·54 ± 0·05

The clear-cut cleavage brought out by the preceding figures suggests three possibilities: (*a*) that the peptone content of brewer's yeast is less than that of Perolin baker's; (*b*) that baker's yeast contains some substance that inhibits the utilization of peptone; (*c*) that brewer's yeast contains some substance that facilitates its uptake. From what we know about the chemistry of the cell, a difference of peptone content capable of explaining the results of varying the quantity of peptone added is intrinsically unlikely. To decide between the two remaining possibilities we added an extract of brewer's yeast to the Perolin medium and determined the effect of varying the quantity of peptone added to it. The extract was a filtrate of a suspension of 10 g. brewer's yeast per 100 c.c. Pearl's salt solution previously put aside for 3 days. The data of this experiment are summarized in table 14.

The results of these experiments lead to two conclusions: (*a*) that peptone itself has no direct effect on the growth of the antennal bud; (*b*) that some substance in brewer's yeast interacts with peptone (or some fraction of peptone) to inhibit its growth.

(*c*) *Fractionation of yeast extracts.* If this is true the peptone series does not necessarily throw light on what constituents of a sterile medium favour exhibition. One way of getting more information of this sort is to study the effect of adding different fractions and different kinds of yeast extract to one and the same sterile medium. The procedure of the first experiment recorded below was as follows. Instead of adding ordinary yeast to the substrate before sterilizing the mixture, we added the dried residue of 10 % suspension of brewer's yeast in Pearl's solution, put aside for 3 days before filtration. Mortality among larvae reared on the extracted yeast medium was not noticeably excessive. Table 15 shows that a cold saline solution removes from brewer's yeast some substance which is essential to the exhibition of the gene. Similar treatment of crude brewer's yeast with ethanol (table 16) has no such effect. The existence of this water-soluble, ethanol-insoluble constituent which promotes exhibition is confirmed by the effect of adding the filtrate of the saline suspension to an ordinary sterile brewer's yeast medium, after shaking it with charcoal (table 17). No such effect follows the addition of saline filtrate to a sterile medium based on baker's (Perolin) yeast. On the contrary the addition of the

TABLE 15

	α	γ
control	0·95 ± 0·02	0·62 ± 0·04
experimental	0·57 ± 0·05	0·15 ± 0·04

TABLE 16

	α	γ
control	0·90 ± 0·02	0·51 ± 0·02
experimental	0·90 ± 0·02	0·50 ± 0·04

TABLE 17

100 % saline		50 % extract, 50 % saline		75 % extract, 25 % saline	
α	γ	α	γ	α	γ
0·87 ± 0·03	0·39 ± 0·04	0·94 ± 0·03	0·50 ± 0·05	1·00 ± 0·01	0·78 ± 0·04

extract produced a fall of the exhibition indices (table 18). We did not treat the filtrate added in the experiment of table 18 with charcoal. So the results suggests that a crude saline extract of brewer's yeast contains a charcoal adsorbable Y substance or substances which inhibit exhibition, in addition to the X substance which is not adsorbed on charcoal. The only obvious loophole in this argument is that the media of the two sets last recorded were not identical.

TABLE 18

control		1 c.c. extract		2 c.c. extract		3·8 c.c. extract	
α	γ	α	γ	α	γ	α	γ
0·87	0·40	0·77	0·30	0·77	0·30	0·66	0·20
± 0·03	± 0·04	± 0·04	± 0·04	± 0·04	± 0·04	± 0·04	± 0·03

TABLE 19. EFFECT OF EXTRACTS (WATER)

brewer's-brewer's extract		control (brewer's)		brewer's + brewer's extract		brewer's + Perolin extract	
α	γ	α	γ	α	γ	α	γ
0·98	0·73	0·99	0·86	1·00	0·91	0·90	0·58
± 0·03	± 0·05	± 0·01	± 0·02	± 0·01	± 0·03	± 0·02	± 0·04
Perolin – Perolin extract		control (Perolin)		Perolin + Perolin extract		Perolin + brewer's extract	
α	γ	α	γ	α	γ	α	γ
failed to come		0·71	0·25	0·84	0·43	0·96	0·69
through		± 0·03	± 0·03	± 0·03	± 0·04	± 0·02	± 0·04

In subsequent investigations of these points we have brought our investigation into conformity with those of Tatum (1939), whose paper appeared during the course of it. Table 19 shows the results of an experiment recording the effect of adding aqueous extract of both sorts of yeasts to media of each type. The aqueous extracts were made in the same way as the saline extracts.

The data of table 19 may be summed up as follows. When added either to baker's yeast or to brewer's yeast sterile medium, an aqueous extract of brewer's yeast increases exhibition. The baker's yeast extract lowers exhibition on brewer's yeast and raises it on a baker's yeast. This disparity confirms the conclusion suggested by the contrast between the properties of saline with or without charcoal adsorption, and therefore suggests the possibility of recovering the inhibitory Y substance (or substances) by first adsorbing it (or them) on to charcoal. Table 20 shows with what success we have been able to do so. It records experiments involving the addition to brewer's sterile yeast medium of Tatum's three fractions of an aqueous brewer's extract. To prepare the fractions we proceed thus:

(i) We shake the extract with charcoal, remove the charcoal with a centrifuge and add the charcoal to the medium. This is fraction I. (ii) Treat the supernatant fluid with barium hydroxide and alcohol as prescribed by Tatum. We separate the precipitate by filtration and wash it with water. The aqueous extract of the precipitate is fraction III. (iii) To get fraction II, we treat the crude yeast with 95% hot alcohol, filter, and allow alcohol to evaporate from filtrate. The residue is fraction II.

TABLE 20. YEAST FRACTIONS ON BREWER'S YEAST

extract added	fraction I		fraction II		fraction III	
c.c.	α	γ	α	γ	α	γ
0	0·96 ± 0·02	0·65 ± 0·06	0·96 ± 0·02	0·65 ± 0·06	0·96 ± 0·02	0·65 ± 0·06
0·5	0·95 ± 0·03	0·59 ± 0·06	0·90 ± 0·04	0·45 ± 0·06	0·97 ± 0·02	0·63 ± 0·06
1·0	0·88 ± 0·03	0·36 ± 0·06	0·83 ± 0·08	0·25 ± 0·06	1·00 ± 0·01	0·58 ± 0·09
2·0	—	—	0·70 ± 0·06	0·36 ± 0·07	0·95 ± 0·03	0·61 ± 0·06
4·0	—	—	0·64 ± 0·08	0·19 ± 0·07	—	—

TABLE 21. YEAST FRACTION I ON PEROLIN

control		experimental	
α	γ	α	γ
0·78 ± 0·04	0·29 ± 0·04	0·66 ± 0·04	0·19 ± 0·04

Table 20 exhibits only a relevant and representative set of data of a much longer series. The figures show significant drop of exhibition due to the addition of fraction I in conformity with that previously stated for the charcoal adsorbability of its Y substance. Even more striking effects of adding fraction II suggest that there may be more than one Y substance, i.e. substance inhibiting exhibition. Fraction III has no effect.

The existence of the charcoal absorbable Y substance is also confirmed by results of experiments in which we added fraction I to a sterile medium based on bakers' yeast (table 21). Whereas addition of the total extract (table 19) clearly *increases* exhibition, addition of the charcoal adsorbate significantly *reduces* it. This leaves no reasonable grounds to doubt the existence of X (*activating*) and Y (*inhibiting*) substances.

(*d*) *The vitamin content.* In view of the high vitamin B content of yeasts, there is good reason to explore the possible identity of one or other of the

B vitamins with substances of these two categories. The following protocol, table 22, records an experiment of this sort. It is clear that B_1, used in quantity in excess of the amount normally present, does not affect exhibition, and B_2, like our Y substance or substances, does so significantly. Ordinarily 1·8–3·0 mg. % of vitamin B_2 is present in brewer's yeast. Baker's yeast contains a higher proportion of vitamin B_2, e.g. 2·5–3·6 mg. % (Fixen and Roscoe 1938). The quantity of vitamin B_2 added experimentally to the brewer's yeast medium was 0·8 mg. %, thus raising the vitamin B_2 (Roche) content to approximately the same level as baker's yeast. The quantity which is significant from this point of view, justifies the conclusion that *difference of B_2 content is largely responsible for the different levels of exhibition on baker's and brewer's yeast media.*

TABLE 22

control		vitamin B_1		vitamin B_2	
α	γ	α	γ	α	γ
0·90 ± 0·04	0·58 ± 0·06	0·99 ± 0·01	0·61 ± 0·03	0·79 ± 0·05	0·25 ± 0·05

10. SUCCESSIVE CHANGES IN THE NORMAL CULTURE

The existence of X substances (which encourage) and Y substances (which inhibit) the exhibition of the gene Antennaless clarifies the paradox of experiments recorded in § 8, i.e. the efficacy of brewer's relative to baker's yeast on sterile and normal media. All yeasts in normal cultures seeded with one or other brands are descendants of a small number of cells cultured under essentially the *same* conditions. If they produce different effects, predominant strains in baker's and brewer's yeast must be genetically different. In the changing environment of a *normal* culture medium containing larvae the brewer's strain produces relatively *more Y and less X*. Brewer's strain added to the *sterile* medium contains relatively *more X and less Y* than the baker's strain. The apparent inconsistency of these two statements disappears if we take stock of one relevant fact. The dead yeasts added once for all to a sterile medium are products of *different* conditions of culture. This must mean that extrinsic circumstances connected with the way in which yeast organisms can be cultured are able to change the relative proportions of X and Y substances which one and the same strain produce.

In the complex sequence of changes which occur in a normal culture we have thus to reckon with two relevant characteristics of the microflora. One is how changing external conditions promote differential fertility and differential mortality of yeast strains genetically distinguishable by their production of X and Y substances in one and the *same environment.* The other is how changing conditions alter the rate of production of X and Y substances by organisms of the *same genotype.* Before we can do this we must get a clearer picture of physical changes which occur in the life of a culture.

(*a*) *Physical state of the medium.* By the third day of a normal medium culture there is a substantial layer of yeast distributed over the surface of the medium. It contains quite large larvae. On the fourth day the surface of the medium in most of the bottles is pitted. The larvae have begun to churn it up to a depth of several millimetres. By the sixth day all but few of the cultures have continuous subsurface air channels throughout the medium. Thereafter the appearance of the surface of the medium varies. This period of surface variation corresponds approximately to the period of variability of its hydrogen-ion concentration; but our data throw no light on the coincidence. At about the ninth day of the culture the medium in some bottles becomes a viscous fluid with no air channels. Moulds may then be present.

Observations made on emergent flies from four sets of cultures throw some light on the relation between exhibition and such medium changes: (*a*) normal medium without nipagin seeded with Hutchison's (wet) baker's yeast (group H); (*b*) *ditto* with nipagin (as above § 8) seeded with Hutchison's yeast (group NH); (*c*) *ditto* without nipagin seeded with Perolin (dry) baker's yeast (group P); (*d*) *ditto* with nipagin seeded with Perolin (group NP). The total number of cultures was one hundred and eleven.

Examination of cultures took place on the fourth, fifth and tenth days after transference of parent flies. On each day we sorted the whole series on the basis of arbitrarily chosen characteristics, as follows:

Fourth day: class I, penetration by larvae of entire surface of medium; II, of part surface; III, no penetration, abundant larvae present; IV, no penetration, few larvae present.

Fifth day: class I, penetration by larvae to a fair depth; II, to slight depth; III, others.

Tenth day: class I, surface definitely wet, no mould; II, not definitely wet, no mould; III, definitely dry, no mould; IV, slightly mouldy; V, markedly mouldy.

For preliminary analysis, we calculated indices based on daily counts of flies in each culture, and made contingency tables to evaluate association between classes in the three groups. The results showed: (*a*) positive correlation between exhibition and moisture in the tenth day group;

(*b*) positive correlation between the arbitrary ratings for larval penetration for individual bottles examined on the fourth and fifth days; (*c*) *no* significant association between ratings for moisture and larval penetration of one and the same bottle. We shall therefore confine the ensuing account to the effect of moisture, i.e. to the tenth day group.

(*b*) *Moisture* (*tenth day group*). Of the four sets of cultures the NH group showed the most nearly random distribution among the five classes obtained on the tenth day. For the descending phase of changing exhibition in the NH group (figure 7), i.e. for the four first days, exhibition for each day's total class count is about the same. For the ascending phase classes I and II rise rapidly at nearly the same rate. Classes III, IV and V are grouped together at a lower level. Figure 8 summarizes data for classes I and III of all groups, i.e. NH, NP, P and H. In all four groups figures for classes I, II and III approximate during the downward phase, figures for the upward phase of class III are uniformly lower than for class II and the latter slightly lower than for class I. The position of classes IV and V relative to the other three classes is less regular. What distinguishes these two classes is presence of mould without regard to moisture. So they are not relevant in this context.

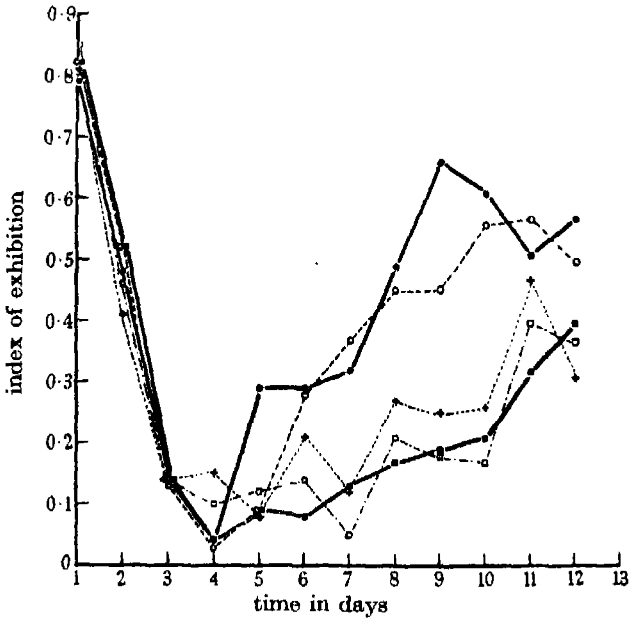

FIGURE 7. Exhibition of NH group: tenth day classification. ● class I; ○ class II; ■ class III; □ class IV; + class V.

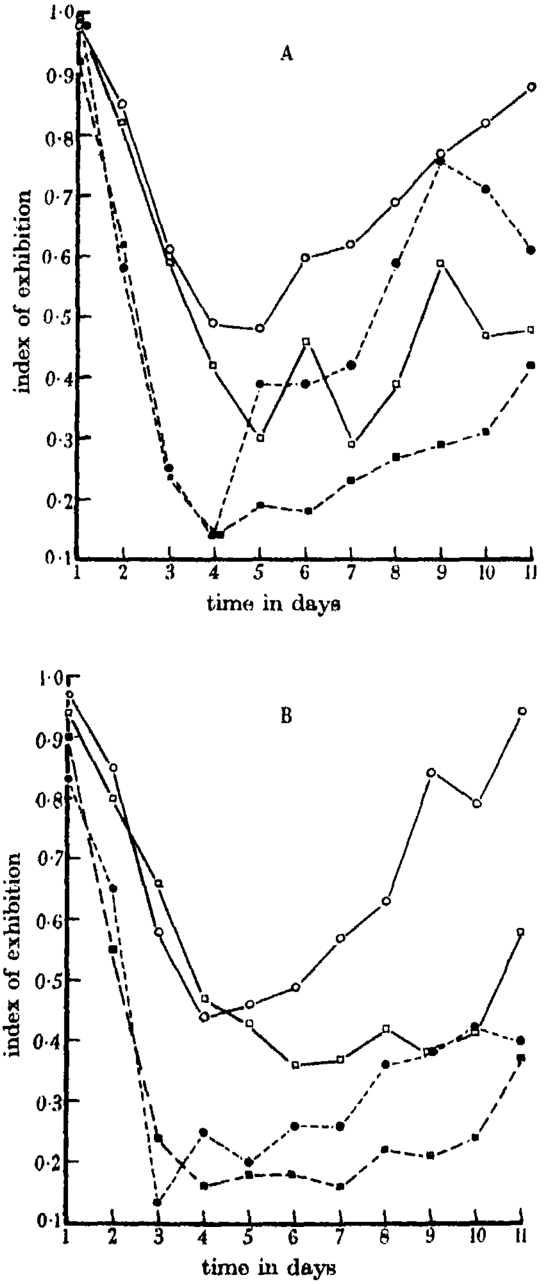

FIGURE 8. Exhibition of all groups: tenth day classification. A, Hutchison's B, Perolin. ○ class I cultures without nipagin; □ class III cultures without nipagin; ● class I cultures with nipagin; ■ class III cultures with nipagin.

Determination of emergence period, i.e. number of days over which the frequency of emergence exceeds ten per culture, showed that the period of emergence in all four groups of class III is less than that of class II. The period of emergence of class II is slightly less than (groups H and P) or the same as (groups NH and NP) that of class I. In cultures assigned to class III the number of flies emerging during (*a*) the first 5 days, (*b*) the first 10 days, (*c*) the whole life of the culture, was less than the number in classes I and II at any time or in any group. The relevant data are in table 23. The graph (figure 8) shows that incidence of exhibition changes in essentially the same way so long as the moisture content of the medium does not fall below a certain limit. If moisture content does fall below this limit the shape of the exhibition-time graph changes profoundly. So also does the

TABLE 23. DURATION OF EMERGENCE AND MOISTURE

	class I	class II	class III	class IV	class V	total
Hutchinson's yeast, without nipagin:						
number of bottles	18	2	3	2	2	27
duration (days) of emergence	15	14	12	10	11	
flies per bottle emerging:						
0–5 days	329	333	289	121	145	
0–10 days	521	510	356	208	221	
0– days	649	586	376	237	236	
Perolin yeast, without nipagin:						
number of bottles	7	5	13	2	0	27
duration (days) of emergence	15	14	12	6	—	
flies per bottle emerging:						
0–5 days	303	258	193	142	—	
0–10 days	496	425	299	173	—	
0– days	626	504	344	179	—	
Hutchinson's yeast, with nipagin:						
number of bottles	3	5	9	6	5	28
duration (days) of emergence	12	12	11	11	11	
flies per bottle emerging:						
0–5 days	356	357	314	265	253	
0–10 days	609	613	465	421	373	
0– days	661	688	541	441	395	
Perolin yeast, with nipagin:						
number of bottles	4	8	7	4	6	29
duration (days) of emergence	12	12	10	9	6	
flies per bottle emerging:						
0–5 days	243	232	254	204	130	
0–10 days	398	436	392	285	158	
0– days	447	460	413	295	158	

emergence frequency distribution. We have now to explore the nature of the relationship between moisture, time-emergence frequency and time-exhibition frequency.

For various reasons, e.g. loss and early death of females or individual egg-laying variability, larval counts of initially uniform cultures may vary widely. This suggests that churning of the medium and hence quicker onset of a wet condition depend on population density. The exhibition time-frequency graphs of wet and dry cultures differ mainly with respect to steepness of the ascending phase. This begins about the same time in both. Our experiments suggest an explanation of the ascending phase. Exhibition increases because the amount of accessible moisture also increases, even in the drier type of culture. While the culture is still honeycombed by tunnels the larvae leave in their tracks, there is continuous circulation of oxygen favouring aerobic respiration of the yeasts. When the tunnels become waterlogged owing to disintegration of the agar gel free circulation of oxygen slows down. So micro-organisms in deeper levels of the culture are forced to anaerobic respiration.

TABLE 24. EFFECT OF NIPAGIN ON STERILE MEDIUM

		nipagin			
	control	0·05 %	0·10 %	0·20 %	0·40 %
α	0·96 ± 0·01	0·96 ± 0·01	0·99 ± 0·01	0·96 ± 0·01	0·95 ± 0·01
γ	0·63 ± 0·04	0·61 ± 0·04	0·66 ± 0·05	0·56 ± 0·04	0·65 ± 0·04
body wt. (mg.):					
males	0·96	0·88	0·88	0·90	0·88
females	1·27	1·25	1·15	1·20	1·05

(c) *Effect of inhibiting fungus growth.* We have now to examine the depressive action (figure 8) of the 'anti-mould' *nipagin*. It has no effect on exhibition frequency or on body weight, when the medium is sterile. So what effect it can have depends on a growing *micro*-population. Table 24 shows this. In the light of results recorded in § 9, these figures mean that nipagin increases the proportion of Y relative to X substances in the medium. We have already shown that the relative proportions of X and Y substances differ in different strains of dead yeasts (§ 8). Since nipagin is an anti-mould its effect on a living culture is presumably due to selective mortality of micro-organisms. It is plausible to conclude that selective mortality of different races of yeasts in a growing culture plays some part in the production of Y relative to X substances, and hence contributes to the character of the exhibition-time graph.

(*d*) *Relation of density to exhibition.* The data incorporated in table 9 suggest that increasing density of larval population increases the steepness of the initial descending phase of the exhibition-time graph for a normal culture. These data refer to experiments in which we varied the *initial* density of the larval population. Others of a different sort point to the same conclusion. We have analyzed exhibition indices on successive days

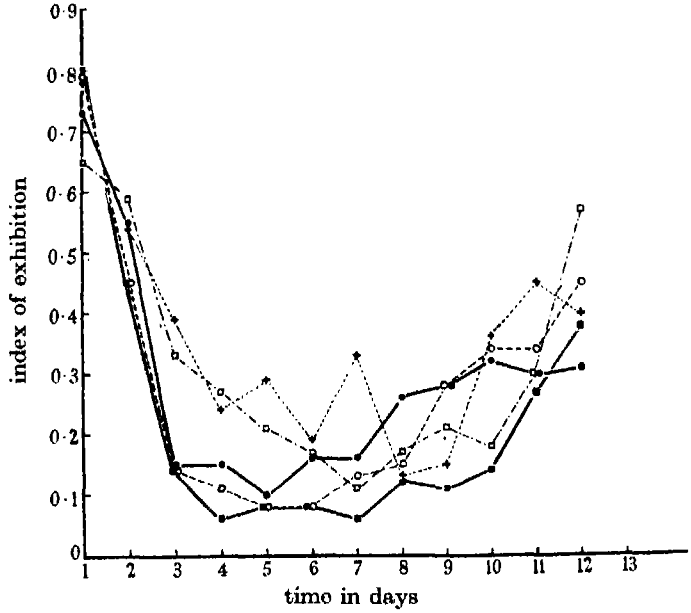

FIGURE 9. Exhibition of NP group: tenth day classification. ● class I, 243 flies per bottle emerged during the first 5 days; ○ class II, 232 flies per bottle emerged during the first 5 days; ■ class III, 254 flies per bottle emerged during the first 5 days; □ class IV, 204 flies per bottle emerged during the first 5 days; + class V, 130 flies per bottle emerged during the first 5 days. This relationship between numbers emerging and the descending phase is the most typical.

in normal cultures containing different types of yeasts in relation to the total number of flies which emerged. Those exhibited in figure 9 (see legend attached) show that the exhibition-time graph falls most steeply during the first 4 days, when the number of emerging flies is greatest. We may take this to mean that decline of exhibition in early stages of the history of a culture depends on some effects initiated by the presence of the larvae themselves.

(*e*) *Life history of a normal culture.* This investigation has raised more issues than it has been possible to solve. As yet they cannot furnish satisfactory evidence for what the authors believe to be a plausible interpretation of the complex sequence of ecological relationships in a live culture containing both growing larval and growing yeast populations. If we make one assumption, we can give a coherent account of successive changes in the life of a culture, as they affect exhibition of the gene Antennaless. This assumption, for which we have some (but not as yet conclusive) evidence, may be stated thus. Aerobic respiration of yeasts favours and anaerobic respiration discourages production of Y substances (§ 9) which inhibit, relative to X substances which promote, exhibition of the gene Antennaless. Our picture of the sequence will then be somewhat as follows. During the first 4 days of a still relatively dry culture the growth of the larval population promotes access of the yeasts to oxygen by creation of tunnels and by churning up the gel. By promoting aerobic respiration of the yeasts this encourages accumulation of Y relative to X substances. So exhibition declines among flies which pass through the *temperature-effective period* during this phase in the life of the culture. Meanwhile the culture becomes increasingly moist. Eventually it becomes waterlogged. A decreasing proportion of yeasts have access to oxygen. More and more they are forced to anaerobic respiration. If the suggestion made above is correct, this means that relatively more X and relatively less Y substances are liberated. Consequently, the latest flies to emerge pass through the *temperature-effective period* during a phase propitious to exhibition of the gene.

The hypothesis stated is provisional. It has at least this merit. It suggests further work for enquiry: (*a*) to justify or to discredit the fundamental postulate, (*b*) if the latter is correct, to explore selective agencies of the culture medium in relation to intrinsic differences between different yeast strains.

11. Discussion

When the expression of a mutant gene of *Drosophila* depends upon conditions of the culture medium, the analysis of its vagaries is an ecological problem. We have to consider how the larval population changes, how the microflora changes and how the metabolites of both, or the constituents of the latter, affect population changes of either sort. In this preliminary enquiry we have preferred to explore a variety of environmental agencies likely to be related to exhibition. Subsequently it may be possible to clarify issues raised in the course of our investigations by: (*a*) further isolation of yeast extract fractions which influence exhibition; (*b*) new information about biochemical idiosyncracies of genetically distinct yeast strains, and of external agencies which promote differential mortality or fertility of the latter; (c) more knowledge concerning the influence larval excretory products or other accumulating constituents of a normal culture may exert on the manufacture of specific substances by yeasts of the same strain.

Meanwhile this much is clear. Even in the highly standardized conditions of a *Drosophila* culture medium the interaction between genetic and environmental agencies is far more intricate than previous work has encouraged us to recognize. Our provisional conclusions are embodied in a model (figure 10) even more complex than the nature-nurture indicator diagram which Hogben (1933) used as a basis for criticizing the application of analysis of variance technique to problems of this type. The issue which Hogben illustrated by using Krafka's data will not be settled by the logical possibility of constructing a model of this sort. Hogben himself recognized this. What is relevant is whether situations of this sort actually and commonly occur in natural populations. If the proportion of clear-cut

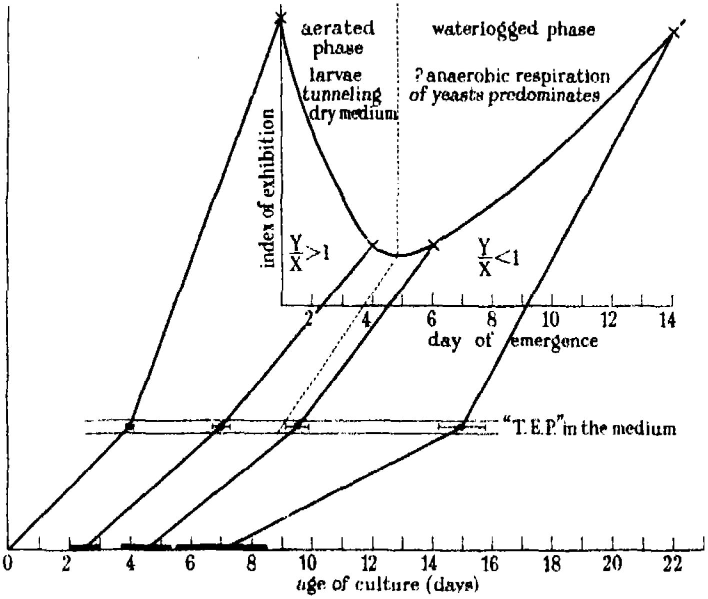

FIGURE 10. Schematic representation of authors' provisional interpretation of events underlying the exhibition-time graph. The exhibition-time graph is shown at the top. The bottom horizontal line shows the spread of oviposition corresponding to a particular day of emergence. The diagram shows that emergence begins about the ninth day of culture. The middle axis showing the spread of the effective period of gene action is arbitrarily placed between the scales for day of emergence and day of culture.

genetically determined traits affected by external conditions in which species live is small, Hogben's criticism, though logically valid, may have scant significance. Whether this is or is not true of human populations, may still be uncertain. Our own experience leads us to believe that exhibition of a *considerable* proportion of mutant genes of *Drosophila*, and possibly of other groups, is likely to respond to changes of diet.

The discovery that availability of a vitamin (B_2) can effectively limit the exhibition of a single gene raises the possibility of undertaking investigations of a different sort. Since Antennaless is remarkably sensitive to small nutritional changes, this mutant may prove to be a serviceable tool: (*a*) for biological assay of accessory food substances; (*b*) for classification of yeasts. Its value as an indicator of interaction between peptone (or some constituent of peptone) and an extract of yeast (§ 8) is also worthy of further consideration because adequate criteria of *in vivo* interaction between specific substances and general food substances are notoriously (Peters 1940) difficult to obtain. Indeed it may be possible to use Antennaless and other mutants with similar idiosyncrasies as test animals to shed new light on problems of insect nutrition.

But for war conditions the authors would have published results of what has been a protracted investigation extending over several years in three stages, independently and successively supported by grants in aid from the Medical Research Council, the Carnegie Trust and the Rockefeller Foundation. Acknowledgements are due to Mrs Frances Gordon—the original discoverer of the mutant Antennaless and of its emergence effect—to Miss E. Souter for the drawings of figure 1, and to Miss J. M. McDonald for technical assistance. We are grateful to Professor Lancelot Hogben for encouragement, advice and editorial assistance with the manuscript, as also to Mr R. M. Neill for criticism.

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The resistance to desiccation of *Drosophila* mutants affecting body colour

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The offspring of a $\frac{y}{y^+} \times y$ cross in *Drosophila melanogaster, simulans, subobscura* and *pseudoobscura* race A showing phenotypically yellow body colour die earlier when starving in dry air (0 % relative humidity and 25° C) than flies of wild type body colour which differ in respect of one gene. The yellow flies also lose more weight.

In *D. melanogaster* F_2 flies of black or ebony body colour survive longer and lose less weight under these conditions than their wild type sibs.

These differences are smaller at 70 % relative humidity, and disappear at 100 % moisture. In the presence of drinking water, yellow females of all four species frequently survive longer than wild type females.

It is suggested that most of these differences in survival time and loss in weight may be due to differences in the resistance to evaporation, which can be assumed to vary with the body colour. This assumption is supported by recent results of Fraenkel and Rudall (1940). Secondary differences due to differences in melanogenesis are discussed.

The ecological meaning of these findings is under investigation.

The ecological meaning of differences in coloration is a problem which has attracted, and is still attracting, many biologists (Cott 1940). Humidity, on the other hand, is one of the most important factors controlling insect life (Buxton 1932). The interdependence of cuticle colour and resistance to evaporation has not as yet been investigated.

Drosophila species offer suitable material for experiments in this respect for various reasons: (1) Flies can be bred in the F_2 generation which grow up under identical environmental conditions, that is to say in the same bottle, and which differ genetically only in a portion of one X-chromosome or one autosome, since all other chromosomes, and parts of that referred to above, are distributed at random. (2) Several degrees of coloration exist in *D. melanogaster* and *simulans*; namely, lighter ones characterized by the absence of black in the body pattern, particularly 'yellow'; and darker ones, for example, 'ebony' and 'black'. In other species the wild type resembles the dark *melanogaster* mutants. The yellow mutants of these species, for example, *D. subobscura* and *pseudoobscura*, correspond to the yellow mutants in *melanogaster* and *simulans*, but not to the wild types.

Thus it appears that in the various *Drosophila* species different more or less corresponding genes have become part of the normal make-up and are obviously more likely to survive in the present environments.

Several papers concerning the survival of mutant *Drosophila* flies under various conditions have been published, and quite recently Greiff (1940) gave a good summary of the results obtained by previous workers. This paper also establishes the greater longevity of an ebony stock as compared with a wild-type stock. A criticism of these results will be found on p. 193.

No experiments have so far been published concerning the longevity in the yellow mutants in the different species of *Drosophila*. In D.I.S. (Drosophila Information Service) the viability of different yellow stocks is reported as sometimes above but usually below that of wild-type flies.

MATERIAL AND METHODS

The following stocks of the four species mentioned above have been used in the experiments:

Melanogaster:	+ oregon	(kept in the Biometry Department,* University College, London)
	y^{39e}	(sent to me by Dr Mather,* John Innes Horticultural Institution, Merton)
	b *eb*	(isolated from a stock sent to me by Dr P. C. Koller,* Institute of Animal Genetics, Edinburgh)
Simulans:	+ *y*	(kept in the Biometry Department, University College, London)
Subobscura:	+ *y*	
Pseudoobscura race A:	+ *y*	(sent by Dr Koller)

The crosses were cultured on the usual agar-maize-yeast medium, *melanogaster* and *simulans* directly in bottles, *pseudoobscura* and *subobscura* being mated in vials and transferred to bottles after one week.

Sibs differing only in the chromosome which contains the body-colour gene were always obtained in the second generation. The procedure was different with black (*b*) and ebony (*e*), which are autosomal genes, and

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* I should like to thank all those workers who have supplied me with stocks.

yellow (y), which is sex-linked. Finally, a combination was used in order to obtain sibs of yellow, normal and black (or ebony) body colour.

The genetical constitutions of the three subsequent generations were as follows:

(a) for b: (the same applies to e):

p: $\frac{b}{b}$ ♀ × $\frac{b^+}{b^+}$ ♂

F_1: $\frac{b^+}{b}$ ♀ × $\frac{b^+}{b}$ ♂

F_2: $\frac{b^+}{b^+}$ ♀, $\frac{b^+}{b}$ ♀, $\frac{b}{b}$ ♀,
$\frac{b^+}{b^+}$ ♂, $\frac{b^+}{b}$ ♀, $\frac{b}{b}$ ♀

Phenotypically three-quarters of F_2 showed the normal body colour, one-quarter was black.

(b) for y:

p: $\frac{y}{y}$ ♀ × y^+ ♂

F_1: $\frac{y}{y^+}$ ♀ × y ♂

F_2: $\frac{y}{y^+}$ ♀, $\frac{y}{y}$ ♀, y^+ ♂, y ♂

Phenotypically half of the ♀♀ and of the ♂♂ in the F_2 were normal and half yellow.

(c) for b (or e) and y:

p: $\frac{y}{y}$, $\frac{b^+}{b^+}$ ♀ × y^+, $\frac{b}{b}$ ♂

F_1: $\frac{y^+}{y}$, $\frac{b^+}{b}$ ♀ × y, $\frac{b^+}{b}$ ♂

F_2: ♀: $\frac{y}{y^+}$, $\frac{b}{b}$, $\frac{y}{y^+}$, $\frac{b}{b^+}$, $\frac{y}{y^+}$, $\frac{b^+}{b^+}$
$\frac{y}{y}$, $\frac{b}{b}$, $\frac{y}{y}$, $\frac{b}{b^+}$, $\frac{y}{y}$, $\frac{b^+}{b^+}$

♂: y^+, $\frac{b}{b^+}$, y^+, $\frac{b^+}{b^+}$, y^+, $\frac{b}{b}$
y, $\frac{b}{b^+}$, y, $\frac{b^+}{b^+}$, y, $\frac{b}{b}$.

Phenotypically the males and females of the F_2 were a mixture of black (or ebony), yellow, normal and black-yellow (or ebony-yellow) flies. The last group was difficult to isolate and was not used in the experiments. The double recessive *y*, *b* is brown in colour.

Two different types of experiment have been carried out: (1) A comparison of the survival of body-colour mutants under conditions of starvation and defined air humidity. (2) A comparison of the loss of weight, principally due to the loss of water, under the same conditions.

For the survival experiments flies which emerged during a specific period were collected from one or several bottles, and kept in a bottle on fresh food. The age of the animals was known and was always fairly constant.

For the experiment proper, groups of twenty to fifty flies were put in vials which were closed with gauze. The vials were kept in desiccators filled with (1) concentrated H_2SO_4 (0 % relative humidity), (2) a mixture of NH_4Cl crystals with a saturated solution of that salt (relative humidity 79·3 % at 25° C) or (3) water (100 % saturation). The vials were lined with filter paper to prevent the flies from drowning in the drops which condensed on the glass at 100 % moisture.

The desiccators were placed in an incubator at 25° C approximately. At intervals of a few hours the number of dead individuals was determined. The loss of weight, due principally to desiccation, was measured under the same experimental conditions, sometimes in the same experiment as the survival. The etherized flies were weighed individually on a torsion balance with a range of 5 mg. showing differences of $\frac{1}{100}$ mg.*

THE SURVIVAL AT DEFINED MOISTURES OF *y*, *e*, *b* FLIES COMPARED WITH WILD TYPE FLIES

It was found in all four species that in dry air yellow flies die earlier than their wild-type sibs. In *melanogaster* black and ebony flies are more resistant to desiccation.

The significance of these differences can be proved by statistical methods. As an example the data for one experiment performed on *D. subobscura* are discussed below.

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* I should like to thank Professor P. A. Buxton of the London School of Hygiene and Tropical Medicine, London, for so kindly lending me this valuable instrument.

Two-day old brothers from one bottle were kept in one vial without food. After 36 hr. at 25° C and 0 % relative humidity the result was as follows:

	alive	dead	total
wild type ♂	12	15	27
yellow ♂	0	32	32
total	12	47	59

If the probability of survival were the same for both kinds, the chance that all twelve survivors would be wild type is $\frac{47!\,27!}{59!\,15!}$ i.e. $\frac{69}{4,443,467}$ or $1{\cdot}55 \times 10^{-5}$. Hence there can be no reasonable doubt that the wild-type flies have a larger probability of survival.

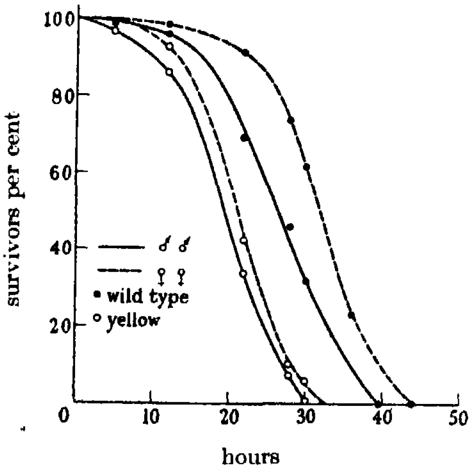

FIGURE 1. Survival of 396 *D. pseudoobscura* flies in dry air at 25° C. The flies were 2–4 days old and the offspring of $\frac{y}{y^+} \times y$ crosses.

The result of a similar experiment, showing the survival of 396 *D. pseudoobscura* flies, is given in figure 1.

In parallel experiments females resisted longer than males, but the difference between wild type and yellow remains. Although undoubtedly significant, it appears to be less in *D. melanogaster* and *simulans* than in *subobscura* and *pseudoobscura*. This is not surprising, because the difference between the normal and yellow mutant in *melanogaster* and *simulans* is only slight, the wild type itself having a yellowish appearance, whereas in *subobscura* and *pseudoobscura* the difference is much more marked. The wild-type colour of these latter species is represented by the mutants black and ebony in *melanogaster*. Figure 2 shows that black-coloured flies are more resistant to desiccation than their wild-type sisters, which in turn are more resistant than the yellow sisters.

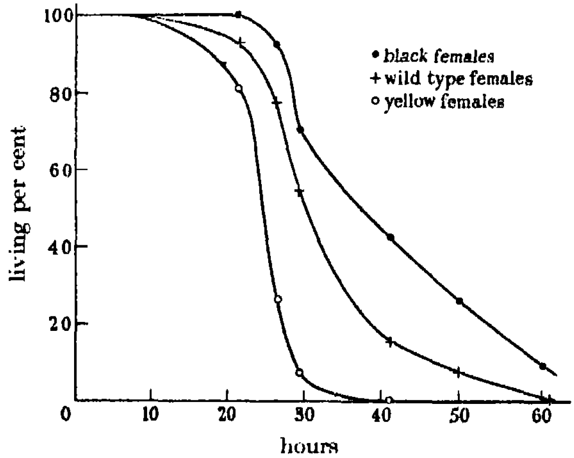

FIGURE 2. Percentage of surviving sisters of black ●, wild type +, and yellow ○, body colours after 21, 26, 29, 41, 50 and 61 hr. starvation in air of 0 % humidity at 25° C.

The following results were obtained in an experiment in which 3-day-old offspring of a $\frac{e}{e^+} \times \frac{e}{e^+}$ cross were subjected to starvation in dry air at 25° C.

original number, sex and phenotype	49 + ♂	19*e* ♂	37 + ♀	21*e* ♀
surviving after 18 hr.	16	10	17	17

The differences in survival of ebony and wild-type flies are just below significance in males ($P = 0{\cdot}21$), but are fully significant in females ($P < 0{\cdot}01$).

At higher degrees of humidity the flies die more slowly, and the difference in the survival of differently coloured sibs decreases. It is still noticeable, if not significant, at 70 % moisture and 25° C, but disappears in the neighbourhood of saturation, especially when condensation drops form, which the flies drink.* As an example of this behaviour, see the data given in table 1.

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* Differences in the survival between wild-type and mutant sibs can also be observed in other mutants. For example, phenotypically vestigial flies die earlier than the wild-type controls. But this difference does not disappear in air saturated with moisture.

TABLE 1. SURVIVAL OF 1–4 AND 5–9-DAY-OLD OFFSPRING OF *D. pseudoobscura A* $\frac{y}{y^+} \times y$ STARVING AT DIFFERENT MOISTURES. $T = 25°$ C

% humidity ...	0 % (H_2SO_4)							
initial age of flies ...	5–9 days				1–4 days			
sex and phenotype ...	y^+♂	y ♂	y^+♀	y ♀	y^+♂	y ♂	y^+♀	y ♀
total number	11	11	17	13	12	12	18	14
alive after 19 hr.	4	0	4	2	12	11	18	11
%	36·3	0	23·5	15·3	100	91·6	100	78·8
alive after 25 hr.	2	0	3	0	11	9	15	9
%	18·1	0	17·6	0	91·6	85·0	83·3	64·2
alive after 43 hr.	0	0	0	0	1	0	4	0
%	0	0	0	0	8·4	0	22·2	0
alive after 49 hr.	—	—	—	—	0	0	0	0
%	—	—	—	—	0	0	0	0
alive after 73 hr.	—	—	—	—	—	—	—	—
%	—	—	—	—	—	—	—	—
alive after 97 hr.	—	—	—	—	—	—	—	—
%	—	—	—	—	—	—	—	—
alive after 115 hr.	—	—	—	—	—	—	—	—
%	—	—	—	—	—	—	—	—
alive after 132 hr.	—	—	—	—	—	—	—	—
%	—	—	—	—	—	—	—	—

% humidity ...	70 % (NH_4Cl)							
initial age of flies ...	5–9 days				1–4 days			
sex and phenotype ...	y^+♂	y ♂	y^+♀	y ♀	y^+♂	y ♂	y^+♀	y ♀
total number	25	23	38	20	36	31	34	15
alive after 19 hr.	20	14	25	14	36	31	34	15
%	80·0	60·8	65·7	70	100	100	100	100
alive after 25 hr.	18	12	23	11	36	31	34	15
%	72·0	52·1	60·5	55·0	100	100	100	100
alive after 43 hr.	1	0	2	0	26	20	25	12
%	4·0	0	5·3	0	72·2	64·5	73·5	80
alive after 49 hr.	0	0	0	0	13	8	15	7
%	0	0	0	0	36·1	25·8	44·1	46·6
alive after 73 hr.	—	—	—	—	3	2	4	2
%	—	—	—	—	8·3	6·4	11·7	13·3
alive after 97 hr.	—	—	—	—	0	0	0	0
%	—	—	—	—	0	0	0	0
alive after 115 hr.	—	—	—	—	—	—	—	—
%	—	—	—	—	—	—	—	—
alive after 182 hr.	—	—	—	—	—	—	—	—
%	—	—	—	—	—	—	—	—

TABLE 1 (*continued*)

% humidity ...	100 % (liquid water)							
initial age of flies ...	5–9 days				1–4 days			
sex and phenotype ...	y^+ ♂	y ♂	y^+ ♀	y ♀	y^+ ♂	y ♂	y^+ ♀	y ♀
total number ...	12	11	19	14	21	19	25	21
alive after 19 hr. ...	12	11	19	14	21	19	25	21
%	100	100	100	100	100	100	100	100
alive after 25 hr.	12	11	19	14	21	19	25	21
%	100	100	100	100	100	100	100	100
alive after 43 hr.	11	9	16	12	21	19	25	21
%	91·6	81·8	84·2	85·7	100	100	100	100
alive after 49 hr.	6	6	7	8	21	19	25	21
%	50·0	54·5	36·8	57·1	100	100	100	100
alive after 73 hr.	6	2	5	6	11	17	22	20
%	50·0	18·1	26·3	42·8	80·9	89·4	88·0	95·2
alive after 97 hr.	0	1	1	1	10	8	7	11
%	0	9·1	5·2	7·1	47·6	42·1	28·0	52·3
alive after 115 hr.	0	0	0	0	1	0	0	1
%	0	0	0	0	4·7	0	0	4·7
alive after 132 hr.	—	—	—	—	0	0	0	0
%	—	—	—	—	0	0	0	0

Two details may be noted from these data:

(1) Older flies survive starvation for a shorter period at all humidities.

(2) Yellow females survive longer at high humidities than wild-type flies. This result is confirmed by the cumulative data from seven experiments, all suggesting the same conclusion. From 312 starving females in 100 % moisture at 25° C the following number survived after various periods:

wild type	73 out of 185
yellow	74 out of 127

The probability that this result is due to chance is less than 0·005. The behaviour of these yellow females will be discussed with other results on p. 198.

WEIGHING EXPERIMENTS

If it is assumed that the differential death-rate of mutants is due to differences in their resistance to desiccation, differences in the loss of water should be observable. These can be roughly measured by the loss in weight.

A determination of the weight of the individuals at the beginning of experimentation is also desirable for another reason. The mutant gene frequently affects several qualities of an individual; and *y*, *b* and *e* in particular can affect body size and weight. In all four species the yellow flies were usually deficient in weight and frequently phenotypically black, and ebony *melanogaster* flies were heavier than the wild-type flies. But sometimes this effect was absent or even reversed. It will be shown later that this at first glance confusing state of affairs can probably be explained simply by the same factor which causes the effects described in this paper, namely, evaporation.

Now it can be shown that larger individuals of the same age and constitution can stand starvation and loss of water significantly longer than smaller ones, and therefore the original weight must be determined. It is desirable that differences in the loss in weight between mutant and wild-type sibs should be tested on samples which do not differ greatly in size (weight),* or the difference should show in the same direction, whether the wild-type individuals are smaller or larger. The size factor was eliminated in most experiments by selecting flies of equal weight after 2 hr. starvation.

Figure 3 shows the differences in loss in weight of batches of phenotypically y^+ and y brothers, descended from a $\frac{y}{y^+} \times y$ cross. In dry air, where the difference shows, simple curves are observed. At first the yellow flies lose more weight, but finally a similar dry weight is reached. At higher humidities complications are noticed, and the difference in loss in weight only appears later at 70 % humidity and is insignificant at 100 % moisture. At 0 and 70 % humidity, death occurs when an individual has lost approximately half its original weight. In saturated air and in the presence of drinking water death occurs before so much weight is lost, obviously for a reason other than desiccation. In the sisters of these flies analogous results were obtained under all conditions, except in 100 % humidity. In moist air *y* ♀♀ lose slightly less weight than + ♀♀. This behaviour corresponds to the result mentioned on p. 192.

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* Greiff (1940) found that the ebony mutant in *melanogaster* shows greater longevity at 80 % humidity and 25° C, but he compared two separate stocks and not the offspring of crosses, and says nothing about the weight of his flies. Therefore his finding, although a confirmation of my results, does not prove that the different body colour due to the mutant *e* increases survival, as it is possible that the *e* flies were bigger. It seems that flies living in the usual culture bottles suffer considerably from evaporation. A few days after hatching yellow flies become deficient in weight, whereas black or ebony flies lose less weight than their wild-type sibs.

The behaviour of the *e* and *b* mutants of *D. melanogaster* in respect of loss of water is the expected one; at low humidities they lose less weight than the wild-type controls or *y*. flies. However, in *e* flies complications sometimes occur in so far as ebony-coloured individuals, especially males, die earlier for extraneous reasons. This is to be expected, as it is well known that many *e* stocks show a rather low viability. It seems that the difference between *b* and b^+ only becomes significant after several days' adult life. Tables 2 and 3 show the average loss in weight of *e* and *b* flies compared with wild-type offspring of the same cross and size. The double recessives $\frac{y}{y}, \frac{b}{b}$ or $y, \frac{b}{b}$ and $\frac{y}{y}, \frac{e}{e}$ or $y, \frac{e}{e}$ have not been considered, because there were not enough of them to give significant results.

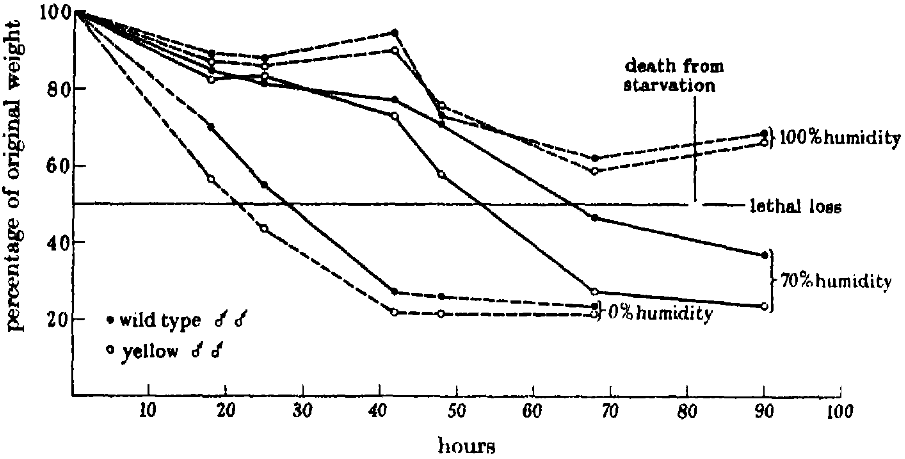

FIGURE 3. Loss in weight during starvation of six batches of six brothers, 2–3-day-old descendants from a $\frac{y}{y^+} + y$ cross in *D. pseudoobscura* race A at three humidities. The level of 50 % indicates the lethal loss in weight.

The single differences between the groups of flies are not significant in themselves except the difference in loss in weight of + or *y* females and *e* females after 24 hr., where P is between 0·1 and 0·05. But as all the differences point in the right direction, the total result can be considered to be significant. A repetition of the experiment, carried out with *e* individuals of larger size, bears this out.

The difference in the loss in weight of black and + females amounting to 8·5 % is significant ($P = 0{\cdot}02$–$0{\cdot}05$). The difference between the males is not significant but points in the right direction.

TABLE 2. AVERAGE LOSS IN WEIGHT OF 1–2-DAY-OLD OFFSPRING FROM $\frac{y}{+}, \frac{e}{+} \times y, \frac{e}{+}$ CROSS IN DRY AIR AT 25° C

number, phenotype and sex	10*y* ♀	10 + ♀	6*e* ♀
weight after 3 hr. starvation at 70 % moisture (in mg.)	0·915 ± 0·044	0·905 ± 0·058	0·743 ± 0·039
loss in weight after 19 hr. (some dead)	0·283 ± 0·043 = 31·3 %	0·259 ± 0·049 = 28·6 %	0·185 ± 0·035 = 27·9 %
loss in weight after 24 hr. (most dead)	0·509 ± 0·057 = 55·7 %	0·467 ± 0·063 = 51·6 %	0·276 ± 0·056 = 37·2 %

TABLE 3. AVERAGE LOSS IN WEIGHT OF 3–4-DAY-OLD OFFSPRING OF A $\frac{b}{b^+} \times \frac{b}{b^+}$ CROSS IN DRY AIR AT 25° C

number, phenotype and sex	7 + ♂	7*b* ♂	8 + ♀	8*b* ♀
weight after 3 hr. starvation at 70 % moisture (in mg.)	0·613 ± 0·017	0·587 ± 0·005	0·980 ± 0·041	0·985 ± 0·033
remaining weight after 14 hr. at 25° C and 0 % humidity (%)	36·59 ± 2·40	38·87 ± 2·46	37·90 ± 2·51	46·42 ± 2·93

DISCUSSION AND ADDITIONAL EXPERIMENTS

The death of an insect which is subjected to starvation as well as to desiccation can be due to either of these factors or to their joint action. This varies in different species (Wigglesworth 1939), and it may also change according to different circumstances. Very little is known of the qualitative changes in metabolism of starving or dying insects, and at present it is impossible to state any general symptom, specific either for starvation or loss of water.

The cause of death in a particular type of experiment must therefore always be investigated independently. The water balance as well as alterations in metabolism must be considered.

The *water balance* of a starving insect shows, as possible sources of gain:

(1) Metabolic water, produced inside the organism.
(2) Adsorbed moisture.
(3) At high humidities condensation droplets or liquid water which is drunk.

Water is lost by:

(4) Evaporation from the tracheal system.
(5) Evaporation through the skin.
(6) Excretion and defaecation.

Of these factors the adsorption of moisture (2) can probably be neglected in *Drosophila*. The quantity of metabolic water (1) produced during the experimental period is small in comparison with the water content of the flies. The drinking of liquid water (3) is the most important gain, its presence or absence deciding whether the water lost by factors (4)–(6) can be replaced or not. Differences in the loss of water can therefore only show in the absence of (3).

According to Wigglesworth (1939) most of the total water lost by insects as a result of evaporation passes through the tracheae (4), the remainder through the cuticle (5). But this may not be true for *Drosophila*. A considerable quantity of water is lost by excretion and defaecation (6). The weight of a single *Drosophila* fly kept on food shows frequent fluctuations up to 30 %. These are mainly due to differences in the content of the digestive system. 2–3 hr. after starvation has commenced, a steady loss in weight can be observed in the absence of liquid water. If drops are available, smaller fluctuations continue. The figures on p. 195 indicate that during the experimental period in dry air the proportion of the total loss of water per hour due to (6) decreases from more than half at the beginning to approximately one-tenth before death.

We need only consider factors (4)–(6) in attempting to explain the different survivals and weight losses of flies of different body colours in dry air on the basis of differences in their water balance. For experiments in moist air, see figure 3.

It is conceivable that mutants differ in digestion or excretion, but there is no indication so far that they lose more water by these activities. On the other hand, the body-colour mutants affect the quality of the cuticle covering both the body surface and most of the tracheal system, as inspection shows. Assuming that a light cuticle offers less protection against evaporation than a darker one, differences in water loss due to factors (4) and (6) could account for the observed differences in death rate and loss in weight.

This assumption is suggested by recent investigations of the insect cuticle by Pryor (1940) and particularly Fraenkel and Rudall (1940). According to these authors the hardening and darkening of an insect, which had hitherto been regarded as separate processes, appear to be only two aspects of the same complex physico-chemical reaction. Any darkening of an insect's cuticle would therefore simply increase mechanical resistance. If this is so, it is only one further step to assume that the permeability for water decreases while the darkening-hardening process goes on. This is strongly supported by details from Fraenkel and Rudall's paper showing that one of the main features of the hardening-darkening process is dehydration during the transformation of the light soft cuticle of a cyclorhaphous fly larva to the hard pupal cuticle. Among others the following changes take place:

(*a*) The ability of the cuticle to swell in water decreases.

(*b*) The water content of the cuticle decreases, and the dry weight increases.

(*c*) The water-soluble fraction of the chitin, and the water solubility of the proteins in the cuticle decrease.

Hardening and darkening of the cuticle of a *Drosophila* fly starts immediately after emergence from the pupa. Wild-type and mutant coloration of the individuals are not acquired by stopping at different points of an identical process, but by qualitatively different processes. Therefore a yellow-coloured fly cannot be compared with a younger wild-type fly. All the colour mutants observed remain darker or lighter during the whole life cycle than wild-type flies of the same age.

In the four observed species the yellow mutants appear softer than their wild-type sibs, but so far attempts to prove this experimentally by means of centrifugal or mechanical pressure have failed. It is probable that the yellow cuticle is more easily wetted than that of the wild-type fly, and this is suggested by the fact that many more yellow flies drown in the condensed water in cultures. Table 4 shows that dried yellow flies take up more water from a saturated atmosphere than their dried wild-type sibs (*D. pseudoobscura*).

TABLE 4

number, phenotype and sex	8 + ♂	8*y* ♂	8 + ♀	8*y* ♀
average weight (in mg.) after 6 weeks' drying	0·237 ± 0·021	0·215 ± 0·031	0·342 ± 0·039	0·331 ± 0·037
average uptake (%) after 24 hr. in 100 % moisture (droplets) and 25° C	67·9 ± 6·0	98·6 ± 9·5	54·1 ± 11·5	78·8 ± 15·3

The differences are significant.

Summarizing all these facts, the physical explanation of the difference in desiccation in *Drosophila* mutants would therefore be as follows: Owing to the different water permeability of the cuticle of the body surface and tracheae in the body-colour mutants, the loss of water differs in dry air but is identical in air saturated with water. Consequently the death of starving flies occurs at identical times in moist air, but light flies die earlier in dry air. For a discussion of the longer survival time of *y* ♀♀ in moist air, see p. 198.

If we assume the validity of this hypothesis, differences in loss in weight must also be expected between dead mutant and wild-type flies. These should be in the same direction as in living flies, but of a lesser degree. The following data support this assumption and hence the whole physical hypothesis.

Experiment 4 *January* 1940. One + ♂, one *y* ♂, one + ♀ and one *y* ♀ were put into each of ten vials. All flies were 3-day-old sibs of a $\frac{y^+}{y} \times y$ cross of *D. pseudoobscura*. After being starved for 3½ hr. at 70 % humidity and 25° C, they were killed with HCN, weighed, and exposed to conc. H_2SO_4 (0 % relative humidity). Table 5 shows the results obtained.

TABLE 5

	+ ♂	*y* ♂	+ ♀	*y* ♀
average weight (in mg.) after 3½ hr. in 70 % humidity	0·868 ± 0·037	0·872 ± 0·038	1·091 ± 0·044	1·093 ± 0·048
average loss in weight after 5 hr. in dry air	0·079 ± 0·011	0·090 ± 0·015	0·090 ± 0·011	0·109 ± 0·011
loss after 28 hr.	0·458 ± 0·068	0·506 ± 0·046	0·596 ± 0·091	0·665 ± 0·058
loss after 45 hr.	0·581 ± 0·051	0·633 ± 0·029	0·774 ± 0·059	0·847 ± 0·051

When subjected to 'Student's' *t* test, most of the single differences in the loss in weight between *y* and + males or females appear to be insignificant. Nevertheless, as all the results point in the same direction, the difference seems to be real. In moist air no significant differences have been observed. Small but probably significant differences after death in the loss in weight between *b* and *e* mutants and their wild-type sibs have also been observed.

So far the experiments on dead flies do not contradict the hypothesis that the different death rate and loss in weight of the body colour mutants is caused by differences in the water permeability of the cuticles. But this hypothesis fails to explain one particular finding, mentioned on p. 192, namely, that *y* females of all four species observed frequently survive longer in saturated air than their wild-type sisters and lose less weight under these conditions. It is inconceivable that, in the presence of drinking water, any movement of moisture through the cuticle could be advantageous to the flies. An explanation of this particular behaviour has not been found. The assumption that more proteins are available for consumption in yellow flies (as they have not combined with polyphenols and so become immobilized) ought to apply for males as well as females, but this is not borne out by experiment. Moreover, in confirmation of Fraenkel's (1936) results on blow flies, *D. pseudoobscura* was kept on a protein-free diet consisting of a 10 % sucrose solution for over 26 days, and neither colour nor sex influenced the time of survival.

As an alternative to the physical hypothesis a chemical concept may tentatively be put forward. Melanism is sometimes regarded as a mechanism for the disposal of toxic phenols arising as metabolic breakdown products (Wigglesworth 1939). It is conceivable that light flies are for some reason unable to dispose of these toxic substances.* In this connexion it is worth mentioning that *y* larvae of *D. melanogaster* are unable to produce black chitin, and they have yellowish brown mouth armatures instead of the normal black ones (Brehme 1937; Kaliss 1937). Obviously the inability to produce melanin remains for life. This larval manifestation of yellow can also be observed in the other three species.

The mechanism leading to death may perhaps be as follows: Desiccation decreases the water content of the flies and the concentration of any toxic substances increases. When a certain level of the poison is reached the flies die. This occurs earlier in light flies than in dark ones. In saturated air, however, this does not happen, and consequently there is no difference in the time of death between *y*, +, and *e* or *b* flies. It has been possible to make a model experiment to demonstrate this pharmacological conception, but no chemical differences could be observed between the body mutants, and in particular no difference in the resistance to phenol compounds. In the model experiment a batch of flies was fed for 5 hr. on 5 % saccharose and 0·5 % CaMg acetylsalicylate, the controls being fed on 5 % saccharose only. After starving in dry air the salicylate flies died much earlier than the control flies. At the time of death the contents of their intestines had lost the power to stain a ferric chloride solution (phenol reaction). In moist air the difference of survival was much less.

The present state of weighing and analytical methods makes it difficult to decide between the two conceptions put forward. But it would seem that the physical hypothesis, ascribing the differences between colour mutants and wild type in death rate and loss in weight to differences in the permeability of the cuticle to water, is far better supported. The peculiar fact that *y* flies frequently survive longer and lose less weight in a saturated atmosphere must, however, be ascribed to differences in chemical metabolism, even if the physical conception is accepted.

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* Graubard (1933) proved the presence of tyrosinase in larvae and pupae of body-colour mutants and wild type *D. melanogaster*, but was unable to find it in the flies. Hence it is very likely that another factor causes the difference in the development of body colours.

Finally, the ecological meaning of the different coloration may be discussed. It is conceivable that in certain circumstances a dark cuticle is advantageous in a dry environment, whereas a light coloration is favourable in moist habitats. In *Drosophila* no ecological facts are known at present which prove or disprove this hypothesis. In general the areas populated by the light species are nearer the equator than those populated by the dark species. This has been shown to be so in Japan by Kikkawa and Peng (1938). In other insects increased humidity tends to produce dark coloration (for example in the cricket, *Gryllus campestris* (Megušár 1913; ref. Hesse, Allee and Schmidt). However, the influence of the single ecological factors on coloration is not uniform. Heat, for example (Hesse Allee and Schmidt), may cause dark colouring, as in individuals of the butterfly *Polyommatus phlaeas* or light colouring as in the species of *Papilio*. The dark colour of many desert animals, particularly insects, seems to provide the strongest evidence for the ecological interpretation of our results. Buxton (1923) points out that 'Any desert creature which is not coloured like its surroundings is black.' Amongst the most remarkable black desert insects are the Tenebrionidae, a family of beetles characterized by hard, dark exoskeletons. Furthermore, other black desert beetles exist, black desert flies of the families Bombyliinae and Anthracinae and black desert grasshoppers, amongst them *Eugaster guyoni*, belonging to a family in which blackness is extremely rare. Buxton also found that a population of another grasshopper *Calliptamus coelesyriensis* which inhabits semi-desert regions of Palestine is composed of two distinct forms, one pale and the other dark chocolate brown. In recent years black desert Tenebrionidae beetles have been found which have the elytra fused with the body wall and have reduced antennae or legs (Hesse, Allee and Schmidt). If we can interpret this as a further adaptation to the arid climate, the hypothesis that black coloration in insects is a 'xerozoic' feature seems more plausible.

Experiments are in progress which are intended to show the influence of different humidities on the composition of populations containing body colour genes.

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The fine structure of collenchyma cells in *Heracleum Sphondylium* L.

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[Plate 8]

The study of *Heracleum* collenchyma follows as a natural consequence of the previous study of sclerenchyma. The cells are elongated with pointed ends much like sclerenchyma cells, but are distinguished from these by their typical collenchymatous thickening, the early onset of thickening, and the heavy impregnation with pectin in the apparent absence of lignin. The thickened wall consists of a series of lamellae (commonly 4–7) running completely round the cell, alternately cellulose-rich pectin-poor and cellulose-poor pectin-rich. The innermost layer of all is almost free of pectin.

The X-ray diagram indicates an orientation of the molecular chains of cellulose approximately longitudinal with an angular dispersion of some ± 14°, and this is fully supported by determinations of the major extinction position. The remarkable uniformity of the m.e.p. suggests that the angular dispersion noted in the X-ray photograph occurs among the micelles in a wall and not among the cells in a bundle used, and the optical discontinuity observed in transverse sections under the polarizing microscope is explained on these grounds. It is suggested that the innermost wall layers are bright in transverse section between crossed nicols because they consist of cellulose micelles with an *angular dispersion* greater than that in layers deposited earlier. The remarkably constant orientation thus revealed in the cellulose-rich layers suggests that either the comparatively few cellulose chains in the layers alternate to them suffice to impose on subsequent layers their orientation or, more probably, that there is in the protoplasmic surface an orienting mechanism itself affected by elongation. The indications are that the longitudinal orientation of the chains in the mature wall, and the more perfect alignment of micelles in the earlier layers, are a consequence of the enormous elongation which the wall has undergone.

Swelling of the wall in a variety of treatments is readily explained in terms of the high pectin content of the central region of the wall and of the greater angular dispersion of the micelles in the innermost layer.

During growth of the cells wall thickening commences long before elongation has ceased, and it is therefore suggested that some modification is necessary of the scheme of nomenclature proposed by Bailey and Kerr. Finally, *at no stage has a wall been observed with cellulose chains oriented even approximately in the transverse direction.*

INTRODUCTION

The present paper represents the third of a series of investigations on the structure of the walls of various types of plant cells. The two previous papers (Kundu and Preston 1940; Preston 1941) were concerned with the lignified fibres associated with the phloem, so that the data to be presented here, relating to a cell type characterized by its high pectin content and apparent absence of lignin, form a contrast with those in the fibre papers all the more interesting since these collenchyma cells, like the fibres, are elongated and thread-like with long tapering ends, and the cellulose component of their walls consists of molecular chains oriented almost longitudinally. The difference between the two cell types is thus largely attributed to their different chemical composition and to their characteristic mode of wall thickening.

The collenchyma cells occur in the form of definite hypodermal strands* in the stem and leaf, particularly in the ribs of the flowering axis, separated from the epidermis by two, or very rarely three, layers of parenchyma cells. Each adult strand tends to be kidney-shaped in transverse section, and is sharply delimited from the surrounding ground parenchyma. Normally each strand is associated with an oil duct opposite the groove on its adaxial side, and with a trace bundle. The collenchyma is a compact tissue with intercellular spaces filled with pectin. Its cells are mostly very long, reaching lengths of some 2·5 mm. with extremely tapering ends terminated by a sharp solid tip (figure 1 (*g*)). This remarkable length, as well as the fusiform shape, makes the cells more equivalent to sclerenchyma than to collenchyma as far as appearance in longitudinal view is concerned; but the typical localized wall thickening (figure, 2, plate 8), the extensibility, the chemical composition, and the manner of deposition of the secondary wall, makes them typical collenchyma. The length and shape of adult cells vary moreover between wide limits. The longest are situated apparently in the centre of the strand, which is the first part to differentiate from the meristem, and the shortest form the flanks added to the strand much later from cells in an advanced state of vacuolation.

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* *Note added in proof.* A detailed description of the development of this collenchyma has already been given by Majumdar (1941).

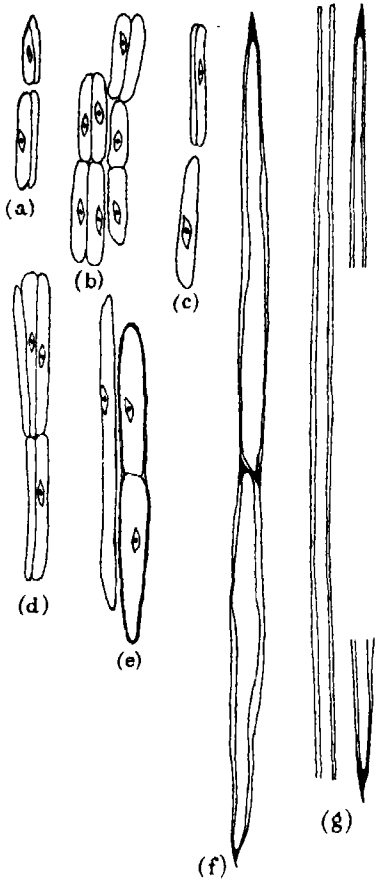

FIGURE 1. Collenchyma cells in various stages of development. (*a*), (*b*) collenchyma initial stage; (*c*), (*d*) mother cell; (*e*) two-celled stage and a mother cell; (*f*) later two-celled stage; (*g*) collenchyma cell. (× 250.)

Each collenchyma cell normally passes through at least three stages before it becomes adult. These we have called the 'initial' stage (figure 1 (*a*) and (*b*)), the 'mother-cell' stage (figure 1 (*c*) and (*d*)) and the 'two-celled' stage (figure 1 (*e*) and (*f*)). The first cell divisions giving rise to collenchyma are always by longitudinal walls which may be in any plane, though predominantly periclinal; these are followed occasionally by transverse division. Such longitudinal divisions may occur a number of times, each daughter cell increasing in volume up to the next division, but it is not until the last division has been completed that any marked elongation begins. This marks the end of the 'initial' stage. The resulting mother cells may develop directly into an adult cell but more frequently, almost normally, they divide by obliquely transverse walls into two daughter cells each surrounded by a complete membrane held together by the wall of the parent cell.* This is the 'two-celled' stage. Further development consists solely of cell elongation and wall deposition, the breadth of the cell tending, if anything, to decrease slightly.

The characteristic thickening, which occurs fairly early in the elongation period, takes the form of a bar along the longitudinal wall at regions where more than two cells meet; the wall in transverse section becomes thickened irregularly. This is preceded, however, by the development of intercellular spaces in these regions which are filled with pectic substances, and this has apparently been mistaken for the first stage in thickening by some investigators (van Wisselingh 1882; Esau 1936). The thickening proper commences rather later than this and our investigations reveal that it takes the form of lamellae running completely round the cell, thicker at the corners.

In the following pages the fine structure of the collenchyma cells in their various stages of growth is studied under the polarizing microscope and, when possible, on the X-ray spectrometer. This part of the investigation is concerned solely with the cellulose component. The distribution of other substances is examined by staining methods and by treatment with various solvents, and their relation to the cellulose complex is examined by treatment of cells, under a variety of conditions, with swelling agents.

A. THE ADULT CELL

(1) *The organization of the cellulose component of the wall*

On the basis of their development, we should expect the walls to have fundamentally a three-layered structure. We shall see later that the actual number of layers revealed by a variety of methods is considerably greater than three, but this should not be allowed to obscure this main principle. It is desirable to know whether these morphologically different layers show any differences in the fine structure of their cellulose component. In the adult cell it is difficult to identify the three layers, though it is possible to separate what is probably the inner thickening layer from the two outer layers, and to show that these latter layers do not differ materially from the inner layer in physical structure.

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* This type of division has already been noted by Priestley and Scott (1939) following an earlier survey of a wide range of growing tissues by Martens (1937, 1938).

Under the polarizing microscope, the wall in transverse section is clearly made up of two layers. In this our observations differ from those of Anderson (1927) on similar tissues in *Solanum lycopersicum*. He found collenchyma cells there to be highly birefringent in transverse section, much more so, in fact, than the surrounding parenchyma. In our material, on the contrary, the layer which is birefringent in transverse section is only feebly so, and appears much less bright than the parenchyma. This difference is undoubtedly connected with the great length of our cells. In addition to this, however, and perhaps of more importance, is the fact that only an inner layer of the wall is birefringent, the outer layer, of considerable extent, being either completely dark or at least of much lower order of birefringence (figure 2, plate 8). A study of longitudinal sections between crossed nicols reveals the fact that this outer layer is actually double. A narrow outermost band is also dark in longitudinal section, and is therefore isotropic or nearly so. This probably constitutes the middle lamella with perhaps those walls present at the two-celled stage. A central layer, however, is very bright in longitudinal section and is therefore markedly anisotropic. The innermost layer of all of a longitudinal bar thickening is in comparison weakly birefringent.

In spite of this discontinuity in optical properties the cellulose chains in the wall appear to run longitudinally only. This is evident both from the X-ray diagram and from major extinction position measurements. The X-ray photograph of a bundle of collenchyma strands is presented in figure 3, plate 8. The photograph closely resembles that of sclerenchyma fibres (ramie, hemp, etc.) in being a typical cellulose fibre diagram. The chains of cellulose run longitudinally with an angular dispersion of some ± 14°. There is no sign of an orientation other than this. Major extinction positions were determined on material macerated in chromic acid and cut open as described elsewhere (Preston 1934), so that observations could be made on single walls. There is no point in giving an extended table of detailed results since the cells are surprisingly uniform. In one representative set of thirty-one cells the maximum inclination of the m.e.p. to the longitudinal was 1·5° and the minimum 0·0° with an average of

0·6° ± 0·08°. Thus the m.e.p. is to all intents longitudinal in the cell. Both X-ray determinations and extinction direction measurements thus agree in demonstrating the presence of longitudinally oriented chains of cellulose. One further point, however, must be noted. The dispersion of the cellulose chains as determined from the X-ray photograph is considerably greater than that of the major extinction position (14° as against 1·5°). This discrepancy is certainly not to be accounted for by dispersion among the individual cells composing the bundle used for the X-ray photograph; it must reflect an angular dispersion among the micelles themselves. This is a point of extreme importance in terms of the observed optical discontinuity of the collenchyma wall as seen in transverse section, and will be discussed later.

(2) *Staining reactions and lamellation of the wall*

In studying the chemical nature and distribution of the non-cellulosic constituents of the wall, staining and solution tests have been made on transverse and longitudinal sections, and also on material macerated by retting in water and by treatment with 5 % chromic acid.

Both transverse and longitudinal sections treated with methylene blue as described in a previous paper (Kundu and Preston 1940) give a pronounced staining reaction for pectic substances. The middle lamella region and outer layers of the wall are stained most deeply. The whole wall, however, contains some pectin, and the indications are that pectin-rich and pectin-poor lamellae alternate. In this we are in complete agreement with Anderson (1927). The pectic substances of the intercellular spaces and middle lamella are easily and completely removed by solvents and the cells, now devoid of any cementing substance, become rounded off and appear isolated in transverse section. A longer treatment is required before the thickened angles of the wall are free of these substances, and as solution proceeds the lamellation of the wall becomes more pronounced. Lamellae are particularly marked in pectin-free cells subsequently treated with a swelling agent (figure 4, plate 8). In transverse sections a light blue cellulose reaction is given with iodine and zinc chloride. Lignin is entirely absent as far as can be judged by staining reactions.

After retting in water, no pectin is to be found anywhere in the walls. Iodine followed by 60 % sulphuric acid gives a pronounced cellulose reaction and the wall is now clearly lamellated, light blue and dark blue zones alternating throughout the whole wall thickness (figure 5, plate 8). 60 % sulphuric acid alone, however, causes immediate solution of the wall. This 'protective' action of iodine against the effect of acid is most marked in our material, though subsequent test on cotton cellulose indicated the same type of action in a smaller degree. We are not sure whether this has hitherto been pointed out.

In material macerated in chromic acid, on the other hand, pectin is still abundant and can be removed by warming in 0·5 % ammonium oxalate. The cellulose reaction is, of course, positive. Here, however, treatment with iodine and zinc chloride produce a series of most peculiar appearances (fig. 10, plate 8). When the macerated cells are treated with iodine first, the bulk of the wall remains unstained. Here and there, however, in the region of the bars transverse blue lines develop. In optical section they appear to commence in the outer part of the wall, but seldom reach the lumen. The blue coloration often spreads very slowly, but it is a matter of hours before the whole wall stains. When the treatment is reversed—zinc chloride first—the whole wall immediately swells and takes on a blue colour. This suggests the presence of thin places in the outer layers of the wall, readily permeated by iodine.

In all these cases, the outer and the innermost layer always behave somewhat differently from the rest of the wall lamellae. The inner layer appears to consist of cellulose comparatively free of pectin. The outer layer always appears quite distinct, and the neighbourhood of an oil duct led us to test its reaction to osmic acid. This layer does, in fact, give faint signs of darkening in this reagent, but hardly so pronounced as to allow any definite conclusion to be drawn. Swelling reactions do suggest that this layer is chemically different.

(3) *Swelling reactions*

The response of untreated material to the action of caustic potash and sulphuric acid is very similar in broad outline to that already reported for fibres (Kundu and Preston 1940; Preston 1941). 20 % potash reduces the walls to a homogeneous glassy mass, with the exception of the innermost wall layer which remains bright and distinct round each cell cavity. Appreciable swelling occurs in sulphuric acid up to 60 % strength, in which the cells dissolve, but again the innermost layer resists solution for a long time. The behaviour of this layer resembles that of the neighbouring parenchyma walls, except in its reaction to zinc chloride. Here only the innermost layer becomes swollen, and the cell lumen becomes obliterated. In transverse sections from which pectin has been removed, swelling takes the following form—inner lamellae swell, become folded inside the stretched outer layer, and close in upon the lumen.

In isolated cells, whether water retted or macerated in chromic acid, swelling proceeds in two distinct steps: (i) a preliminary uniform swelling of the cell as a whole, with the outer layer intact, and (ii) a further swelling after special treatment, often associated with rupture of the outer layer. The same two stages are of course invariably a feature of swelling even in the fibres, but here they are unusually pronounced.

(i) *The preliminary swelling.* This initial stage in swelling is accompanied by a pronounced decrease in cell length, and the wall becomes very transparent. In the water-retted cells the maximum preliminary swelling seems already to have occurred. These cells are always much shorter and wider than comparative cells obtained by maceration, as the following figures will show. They represent averages of thirty cells taken from the same collenchyma strand.

	length	breadth
macerated in 5 % chromic acid	1590 ± 77 μ	12·85 ± 0·50 μ
water retted	1299 ± 51 μ	15·60 ± 0·65 μ

Together with this swelling the cells lose their firmness; they are limp and form entangled masses quite unlike the macerated cells. In many cases the lamellae are distinct along the longitudinal bar, and appear to vary from four to seven in number. No further general swelling occurs to any marked extent in any reagent, though in zinc chloride a slight swelling may be presumed since the relatively inelastic outer layer is often thrown into folds.

Chromic macerated cells are therefore the more suitable for a study of preliminary swelling. Here, caustic potash in strengths up to 10 % causes slight general swelling, but no lamellae are visible. In strengths of 20 %, however, we appear to get maximum preliminary swelling comparable in detail to that produced by retting in water. The cells are now broader and shorter and are limp. In appearance, therefore, they are indistinguishable from water-retted cells. The outer layer is often thrown into very close folds, giving the cells as a whole a pronounced wavy outline (figure 8, plate 8). Zinc chloride produces swelling of the same order, but also initiates a slow progressive solution of the wall, beginning at the outside, until after about 15 hr. the whole wall is dissolved except the innermost layer. Irrigation with iodine causes this inner layer to assume the blue coloration typical of cellulose. The inner layer thus appears to be the most resistant to zinc chloride. In sulphuric acid up to 57 % the wall swells considerably and the cells shorten. Here, however, folds develop both in outer and inner layers, indicating a more generalized resistance to swelling in sulphuric acid than in zinc chloride. This resistance of the inner layer to acid is, however, only temporary, for prolonged immersion in 57 % sulphuric acid causes almost complete obliteration of the lumen. Reaction to sulphuric acid is therefore in some respects opposite to that in zinc chloride.

Maximum preliminary swelling thus occurs in cells either (1) water retted, or macerated and treated with (2) saturated zinc chloride, (3) 20 % caustic potash or (4) 57½ % sulphuric acid. In spite of the similarity in swelling, however, only when iodine is added to (2) or (4) does the wall appear blue. This would suggest that the similarity is merely superficial; and since we know that treatments (2) and (4) cause a swelling of the cellulose component, it might be assumed that water retting or treatment with caustic potash affects cell dimensions through changes in the pectic component. This is borne out by the fact that collenchyma bundles retted out in water without drastic separation of the cells give a typical X-ray fibre photograph of natural cellulose in spite of their limp and flabby nature.

(ii) *Further swelling after pretreatment.* Prolonged treatment with zinc chloride or with strengths of sulphuric acid of 60 % or more leads eventually to complete solution without deformation as a unit. While this solution is also to be classed as swelling in the chemical sense, we here prefer to limit the term to changes in volume while retaining coherence. Swelling beyond the preliminary stage occurs only if the cells are protected against the solvent action of the swelling agents. Such protection is afforded by aqueous iodine.

The type of swelling seems to depend on the nature of the outer layer. If this becomes cracked transversely the swelling takes the form of typical balloons described for cotton, hemp and other fibrous cells. If this layer cracks along a steeply spiral line swelling is continuous and takes a twisted spiral form with the split outer wall appearing as spirally wound bands. Both water-retted and macerated cells treated with iodine and immersed in zinc chloride present the former appearance. The overstretched outer layer cracks transversely or occasionally in close spirals and the swelling layers inside protrude (figure 6, plate 8). The segments of the outer layer then 'slide' down these protuberances and collect at the constrictions as transverse bands. The deep blue collar thus developed is now negatively dichroic, whereas the original segment was positively so. Pretreatment with caustic potash results in a much more swollen cell in which lamellation is beautifully clear, but the balloons eventually go into solution.

60 % sulphuric acid after iodine, on the other hand, induces predominantly the second type of swelling reaction (figure 7, plate 8). Inner

layers swell considerably and assume a deep blue coloration. This swelling results again in a series of fissures in the outer layer, which, however, this time are longitudinal or steeply spiral instead of transverse. Here again, therefore, the reactions of the wall to zinc chloride and to sulphuric acid are strongly contrasted.

B. THE DEVELOPING CELL

Perhaps the most striking feature in which the collenchyma of *Heracleum* differs from sclerenchyma lies in the early stage at which wall thickening commences in cells of the former. The relevant data are summarized in table 1. Thickening, rarely seen before the two-celled stage, becomes a predominant feature after the division of the mother cells. It is from this point on that the bulk of the extension occurs (up to 2600 % of the original mother cell size). While this enormous extension is taking place, the internode as a whole is also elongating at about the same rate (table 1), and though it is not impossible that this is associated with equal but independent growth rates of both collenchyma and surrounding parenchyma, it seems far more likely that the growth rate of the collenchyma strands is conditioned by that of the parenchymatous tissue in which it is imbedded.

TABLE 1. COLLENCHYMA CELLS AT VARIOUS STAGES OF GROWTH

plant	length of internode, *I* (cm.) (flowering axis) cm.	cell dimensions (μ)		*I*/*C**	stage of development	remarks
		length (*C*)	breadth			
1	0·5	64·6	7·14	—	mother cell	no intercellular spaces
	0·8	95·6	8·35	—	mother cells elongated	intercellular spaces filled with pectin
	1·4	104·5	8·08	—	a few two-celled stage or early collenchyma cell	a few cells thickened at corners
	1·6	150·1	8·67	—	two-celled stage	regular collenchymatous thickening
	26·5	174·8	11·9	—	collenchyma cells	,,
2	4·9	246 ± 10	14·2 ± 0·3	199	,,	,,
	26·5	1155 ± 33	15·3 ± 0·4	230	,,	,,
	24·8	1275 ± 53	13·6 ± 0·4	195	,,	,,

* This column is completed only in the lower part of the table, where a number of cells was measured sufficient to allow a significant mean to be derived.

The collenchyma may, in fact, be extending passively under the tensions imposed upon it by the rest of the tissues in the stem. Now we know roughly how cell walls behave under such extension from studies of various cell types (Preston 1934, 1938, 1939; Astbury and Preston 1940), and it should therefore not appear surprising that in adult collenchyma the cellulose chains run almost longitudinally in such a uniform manner.

Study of very young stages in development lead us to suspect that this straightening out of the molecular chains has commenced even earlier than might be anticipated. We have examined cells as early as the two-celled stage, but it is unfortunately impossible with our present technique to slit open such delicate cells without serious deformation. We can therefore not be definite upon this point. The appearance of whole cells is, however, suggestive. In macerated tissue, the wall in face view (double wall) is feebly anisotropic and in edge view more strongly so. In both cases the double refraction is *positive* with respect to the cell axis. In no case have we observed a cell with negative double refraction. No useful purpose would be served by presenting detailed measurements of the strength of double refraction here. The fact itself surely means that the cellulose chains in the individual walls are inclined to the longitudinal axis of the cell at angles less than 45°. While chains more nearly transverse, and perhaps completely transverse, may occur at earlier stages, or may even occur at the two-celled stage in very exceptional cases, we have never seen any cell in which we might suspect that the inclination was flatter than 45%.

The walls at this young stage may provisionally be called *primary*. *Here again, then, we have another extending wall in which cellulose chains make a considerable angle to the transverse plane.*

We may imagine this young delicate wall, consisting probably of cellulose chains running in moderately flat spirals imbedded in substances of a pectic nature, undergoing this enormous extension during which the chains are gradually pulled out into steeper and steeper spirals. Now the major part of the extension occurs long after the onset of wall thickening, and this fact again brings up the question of nomenclature in wall development. It is not easy to fit collenchyma into the scheme proposed by Kerr and Bailey (1934), in which the wall surrounding the original meristem cell, capable of extensive plastic deformation, is defined as *primary* while later, less extensible layers deposited after cell enlargement has ceased are denoted *secondary*. In collenchyma, the layers of thickening are deposited long before extension has ceased. They cannot be termed primary, for primary walls are not considered to undergo any considerable irreversible changes in thickness; the term secondary hardly covers them either, for they are definitely extensible, and while other cases of secondary walls are known which are suspected to undergo extension (Preston 1939), the changes in dimension involved are of an entirely different order. The nomenclature of wall morphology proposed by Kerr and Bailey is thus much too rigid to include types like collenchyma. The wall up to the two-celled stage is perhaps primary, but the thickening layers deposited after that stage are, at best, certainly not secondary, but perhaps a special case of primary. The confusion arises from the sharp line implied between extension and non-extension. Wall deposition is surely a continuous function of time, and it is by no means clear why the metabolic factors underlying deposition should change equally with those underlying cell enlargement. The cytological and ontogenetic grounds for the classification of Kerr and Bailey are clear; the physiological basis is more obscure. We clearly have to visualize not two but at least three conditions. If a cell is extending with no obvious changes in wall thickness then we may, with some justification, refer to the wall as primary; when the cell has ceased to extend, subsequent depositions are, with equal clearness, to be called secondary. This distinction covers the majority of cell types very satisfactorily. But now in some cases we must visualize a condition in which the controlling metabolic factors have initiated rapid wall development without a cessation of cell enlargement, a condition which possibly occurs for a very brief period in all cells and is exaggerated in a few types like collenchyma. We feel that the necessity for this third condition requires some modification of the existing classification, and in view of the long-established usage of the terms primary and secondary this modification should preferably involve the introduction of a third term. We suggest the term *thickened primary*.

DISCUSSION AND CONCLUSIONS

The wall of collenchyma cells thus consists of many well-defined concentric layers running completely round the cell. In the thickened regions these layers, as first pointed out by Anderson (1927), are alternately cellulose-poor pectin-rich and cellulose-rich pectin-poor. The molecular chains of cellulose run almost longitudinally in all cellulose-rich layers, and this longitudinal arrangement arises as a natural consequence of the enormous elongation which the cell has undergone. This brings the collenchyma of *Heracleum* into line with the sclerenchyma already studied in this series. In the present case the connexion between wall structure and elongation is perhaps more clearly expressed even than in sclerenchyma, since in other cells of the same type, differing from our material only in their lower degree of elongation, the chain orientation appears to be of an entirely different type (Anderson 1927). This universally longitudinal orientation of cellulose chains in a markedly lamellated wall is of particular interest. The layers we have called cellulose-poor are in fact so lacking in a coherent cellulose skeleton that the alternate cellulose-rich layers often fall apart when pectin is removed from the wall. They approach the cellulose-free condition, and it is therefore a point of importance that the chain orientation remains constant throughout the cellulose-rich layers. In a wall where we might expect the crossed fibrillar structure suggested for a number of cell types, the phenomenon is completely absent. Certainly the last layers laid down are bright instead of dark in transverse section under the polarizing microscope, but it seems clear that this is not a consequence of any change in the *general* direction of the chains. It is our experience that this layer appears only in cells from mature internodes or from internodes becoming mature, and we suspect that they are deposited after cell elongation has ceased. It would appear significant that while the m.e.p. of the whole wall (which measures the mean orientation of the cellulose micelles) varies only within narrow limits (0·0–1·5°), the dispersion indicated in the X-ray diagram is considerable (± 14°). These two facts suggest that the cellulose chains are dispersed symmetrically about an axis not far removed from the longitudinal, and if we assume that this angular dispersion occurs largely in the inner wall layers, then the brightness in optical cross-section is explained. The evidence points to a greater *angular dispersion* in the inner wall layers, a conception which is feasible if these layers, unlike earlier ones, are not subjected to passive extension after their deposition. They would represent the condition of a layer as laid down by the cytoplasm; the more perfect alignment of chains in earlier layers would then be the result of extension. This is supported by the fact that the boundary between bright and dark layers is diffuse, as it should be if new wall material is continuously deposited during the slowing down of the elongation process. The appearance of a maltese cross on the bright layer viewed between crossed nicols suggests that the dispersion is confined to the tangential plane, and on these grounds we propose that the organization of the collenchyma wall studied here may be represented as in figure 9. We suggest further that while, during development, change in micellar orientation is governed by change in cell dimension, the orientation in layers already deposited is imposed on new layers either in virtue of an orienting mechanism residual in the comparatively few chains in the cellulose-poor layers, or because of a mechanism in the cytoplasmic surface itself affected by elongation.

Optical discontinuity of the present type has now been observed in a number of cell types, and in one other case (Preston 1939) is again clearly connected with changes in the angular dispersion of the constituent micelles. The same principle probably applies also to the fibres of hemp (Kundu and Preston 1940) and of jute (Preston 1941). In the former case, the arcs of 3·9 A spacing observed in the X-ray diagram of a single fibre (figures 5 and 6, plate 14, Kundu and Preston 1940) are largely confined to an angle of ± 5°, which corresponds roughly to the dispersion to be expected from the steeply spiral arrangement of the m.e.p. The arc can, however, be traced over a further ± 5° (approximately), and this further dispersion can only be due to an actual angular dispersion among the cellulose micelles. In jute the condition is rendered much less clear owing to the rather wide variation of the m.e.p. from cell to cell. Here the dispersion in the X-ray photograph is fully accounted for by the dispersion

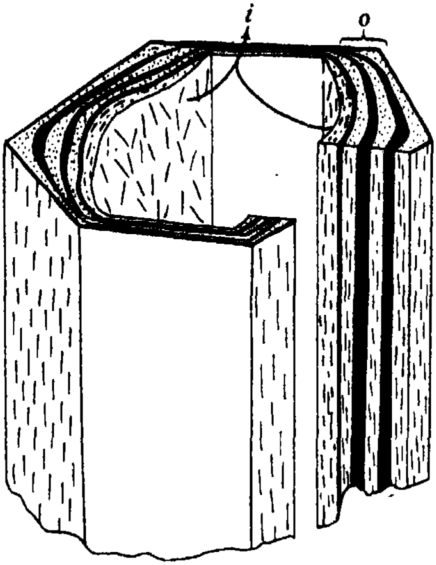

FIGURE 9. Diagrammatic representation of the structure proposed for the walls of *Heracleum* collenchyma cells. Cellulose-poor layers are blackened. In the rest of the wall the cellulose micelles are represented by short narrow rods, and therefore in the bulk of the wall (outer layers, *O*) appear as short lines in longitudinal view and as dots on the transverse face. The angular dispersion in the inner layers, *i*, causes the appearance, in perspective, of very much shortened rods on the transverse face. This layer only, therefore, is anisotropic in transverse section.

of the m.e.p. among the cells. At the least, however, we may say that the results of X-ray analysis furnish no evidence against the change in angular dispersion as suggested for hemp and for the collenchyma cells studied here, and the absence of any chains with a more or less transverse orientation again leaves us with only such a change in dispersion to account for the observed optical discontinuity in cross-section.

It is not surprising that the walls of collenchyma cells are so readily swollen in view of their high content of pectic substances. By comparison of the present cell type with sclerenchyma we may say that pectin increases, and lignin decreases, the response of a cellulosic wall to swelling agents. The response of these pectin-rich walls to zinc chloride and to sulphuric acid is, however, somewhat peculiar. The results are summarized briefly in table 2. It is to be remembered that in the untreated cell the innermost layer differs from other layers in at least two respects. This layer is much poorer in pectin than is any other layer, and the micelles of its cellulose complex appear to have a greater angular dispersion.

TABLE 2

	relative swelling* of wall layers in			
	zinc chloride		sulphuric acid	
condition of walls	inner layer	central layer	inner layer	central layer
untreated	+ +	+	+	+ +
chromic macerated	+	+ +	+	+ +
pectin-free	+ +	+	+ +	+

* The number of + signs indicates the relative swelling of the layers.

It seems clear that both conditions play a part in determining swelling behaviour. Sulphuric acid apparently induces such swelling of the pectic substances in the untreated and macerated cells that this overrides any specific effect of the configuration of the cellulose itself. When pectin is completely removed, however, the greater dispersion of the cellulose matrix in inner layers allows a more rapid penetration of the swelling agent and therefore a more rapid and extensive swelling. With zinc chloride this latter process prevails whether pectin is present or not, except with cells previously treated with chromic acid. In view of the fact that chromic acid is a strong oxidizing agent, which may therefore modify either the cellulose or the pectin or both, this exception would appear to be of minor importance. The low resistance to swelling of a layer whose cellulose micelles have a greater angular dispersion recalls a similar feature in the jute fibre. There lignin is readily removed from the layer in question (which this time is an *outer* layer, but is still probably the first layer deposited after cessation of cell elongation), and the layer is then less resistant to swelling than any other layer in the wall. This is true even if lignin is entirely absent from the rest of the wall, and is therefore a specific effect of the organization of cellulose. In the case of hemp the heavily impregnated outer layer is difficult to free of lignin without the use of drastic methods, so that this layer remains largely resistant to swelling in spite of the angular dispersion of its constituent micelles.

Finally, in collenchyma as in the sclerenchyma of hemp and jute we have a clear and convincing case of an extending wall composed of cellulose chains oriented almost longitudinally. In view of the improbability that the chains in the walls of all collenchyma initial cells are similarly oriented, the conclusion is inescapable that during extension a change in chain orientation occurs.

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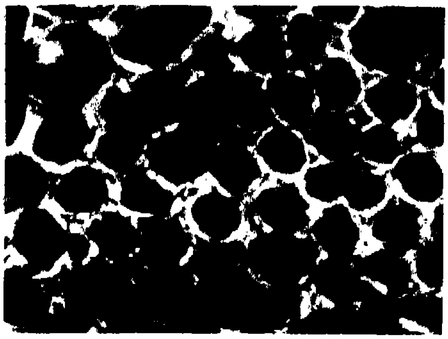
EXPLANATION OF PLATE 8

FIGURE 2. Collenchyma of *Heracleum* in transverse section under the polarizing microscope, showing the bright inner wall layers and the thicker, dark outer layers. Note the characteristic irregularity in wall thickening.

FIGURE 3. X-ray diagram of a bundle of collenchyma cells (bundle parallel to the longer edge of the page). Note that the strong arcs of spacing 3·9 A and of 5·4 and 6·1 A occur on the equator only. There is therefore evidence of no orientation other than longitudinal.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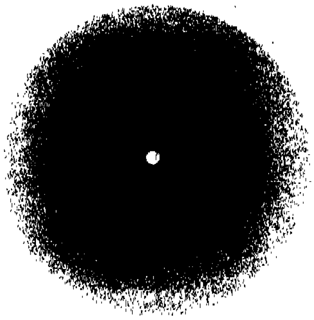
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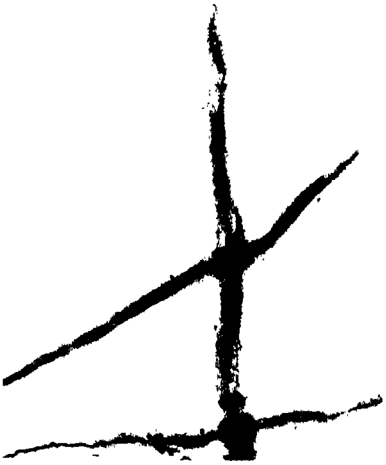
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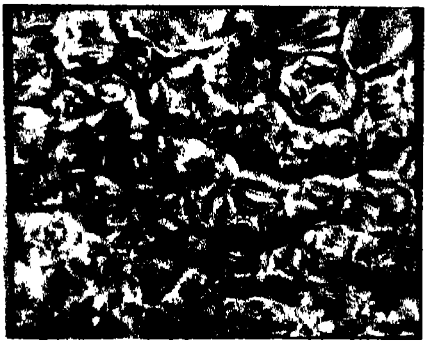
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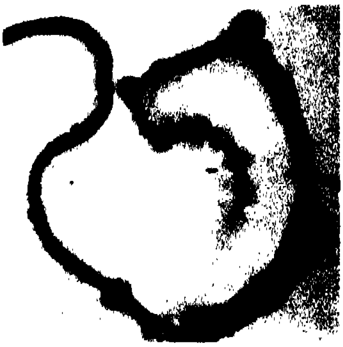
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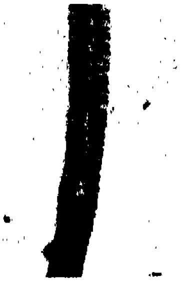
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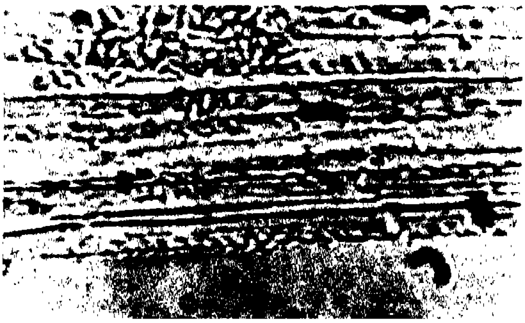
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FIGURE 4. Collenchyma in transverse section after removal of pectin. Note the prominent lamellation. (× 650.)

FIGURE 5. Collenchyma in transverse section treated with aqueous iodine and 60% sulphuric acid. Note the pronounced staining of alternate layers. (× 230.)

FIGURE 6. Ballooning in collenchyma treated with iodine followed by zinc chloride. Notice particularly on the right the lateral bursting of the outer layer and the protrusion of the swelling central layer. (× 250.)

FIGURE 7. Collenchyma cells swollen in sulphuric acid after treatment with iodine. The outer layer has split in a steep spiral and swelling is almost uniform. (× 170.)

FIGURE 8. Collenchyma cells treated with 25% KOH. Note the prominent folding of an outer resistant layer. (× 140.)

FIGURE 10. Macerated collenchyma treated with iodine followed by $ZnCl_2$. (× 140.)

The behaviour of visual purple at low temperature

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Visual purple is soluble and stable in a mixture of glycerol and water (3:1). At room temperature the spectrum of such a solution is identical with that of the aqueous solution. At −73° C the peak of the absorption curve is higher and narrower than at room temperature, and it is shifted towards longer waves.

The product of photodecomposition at −73° C has a spectrum independent of pH and is at low temperatures thermostable and photostable, but at room temperature it decomposes thermally to indicator yellow. The primary product appears to be identical with transient orange.

The quantum yields of the photoreaction at low and at room temperature are of the same order.

Introduction

Numerous studies have been devoted in recent years to the absorption spectrum and photochemical kinetics of visual purple, the chromoprotein contained in the 'rods' of the retinae of vertebrates. In this paper are described experimental studies at low temperatures up to the point where they were interrupted by the war.

Previous to this work only aqueous solutions of visual purple have been prepared and consequently no solutions could be investigated below 0° C. It has now been found possible, however, to extend the range for experiments on visual purple solutions by the use of a solvent consisting of 75 % glycerol and 25 % water by volume. Such solutions assume glass-like consistency at the temperature of solid CO_2 and remain perfectly homogeneous and brilliantly clear. Even at liquid-air temperatures no crystallization occurs, although a great number of minute cracks due to the contraction of the mass scatter the light and prevent spectroscopic investigations.

While small amounts of any other organic solvent soluble in water to some extent, e.g. alcohol, ether and chloroform, bleach aqueous solutions of visual purple and produce 'indicator yellow', even large quantities of glycerol do not harm the chromoprotein. The peculiar toleration of visual purple for glycerol recalls results obtained with 'serum protein' (Spiro 1903). The stability of aqueous solutions of this protein against denaturation was decreased by the addition of univalent alcohols, especially of higher molecular weight, but was increased by polyvalent alcohols, e.g. glycerol, mannitol, glucose, galactose and lactose.

THE ABSORPTION SPECTRUM

Experiments were carried out to determine whether visual purple belongs to the wide class of coloured organic substances, the absorption curves of which sharpen on reduction of temperature.

Experimental

The spectroscopic determinations were carried out in a room illuminated with ruby light with a Hilger Spekker photometer. The light beams were produced by an iron spark, and filtered through an acid quinine sulphate solution in order to eliminate ultra-violet and violet light. Hypersensitive panchromatic Ilford plates were employed, and the use of a strong metol developer facilitated short exposures. No appreciable decomposition of visual purple occurred during the spectrographic determinations.

The visual purple solutions were prepared from frogs' eyes as described by Lythgoe (1937). Enough glycerol and water were added to the buffered solutions to obtain a mixture containing 75 % glycerol and to fill the all-glass optical cell 5 cm. long and 2·7 cm. wide. The optical density of the solution so obtained at 500 mμ was of the order 0·2 cm.$^{-1}$. The cell was fixed into a rubber-lined brass box lagged outside with asbestos. The box served to hold the mixture of solid carbon dioxide and alcohol, thus keeping the temperature in the cell at −73° C. Two holes facing the cell ends were drilled into the walls of the box so that the light beam could pass; the diameter of the holes was slightly less than that of the cell. One of the two walls provided with holes was detachable and could be screwed to the rest of the box, after the cell was fitted in, in order to hold the cell firmly in position. The rubber lining on both holed walls of the box acted as washers.

In order to prevent condensation of water vapour on the faces of the cell, glass cylinders closed with small plates of optical glass were fixed with tap grease coaxially to each end of the cell and supported by joining them to the metal box with apiezon grease. The optical glass plates were sealed to the cylinders with apiezon, and heated electrically by means of wire spirals wound around the cylinders. The cylinders were kept evacuated during experiments. In this way all glass faces remained perfectly clear. The comparison beam passed through a cell containing solvent only. It was not cooled, but preliminary tests showed that the zero of the spectrophotometer was maintained when the cell in the box, filled with pure solvent, was cooled.

Mist was formed near the cold box, but a powerful fan operating towards the light beams dispersed it and prevented interference with the beams.

Results

The first measurements showed that the spectrum of visual purple in glycerol-water solution at room temperature is identical with that of a twin solution diluted with water to the same strength. Furthermore, it was found that the spectrum remains unaltered by cooling the solution down to −73° with exclusion of light and warming up again to room temperature.

In figure 1 the spectrum of a solution (pH 6·0) at room temperature (curve 1) is compared with that at −73° (curve 2). Curve 1 comprises densities both in water and in glycerol-water. The influence of thermal contraction, which is very nearly 10 %, was eliminated by subtracting 10 % from the density values obtained at the low temperature.

Curve 2 is not only narrower and higher than curve 1, but the peak is at about 515 mμ instead of at 505 mμ. A similar shift in the position of an absorption peak on cooling was observed with solutions of diphenyl-polyenes (Hausser, Kuhn and Seitz 1935) and of β-carotene (Bowden 1934). On the other hand, solutions of porphyrins and related compounds, including oxyhaemoglobin, show a shift in the opposite direction (Hartridge 1921; Hellström 1931; Conant and Kamerling 1931). It is remarkable that the spectra of the compounds with the shift to shorter waves are of one particular type; they all show sharp maxima and steep thresholds. The meaning of the difference in the shape of the bands has been discussed by Arnold and Kistiakowsky (1932) and by Goodeve (1939).

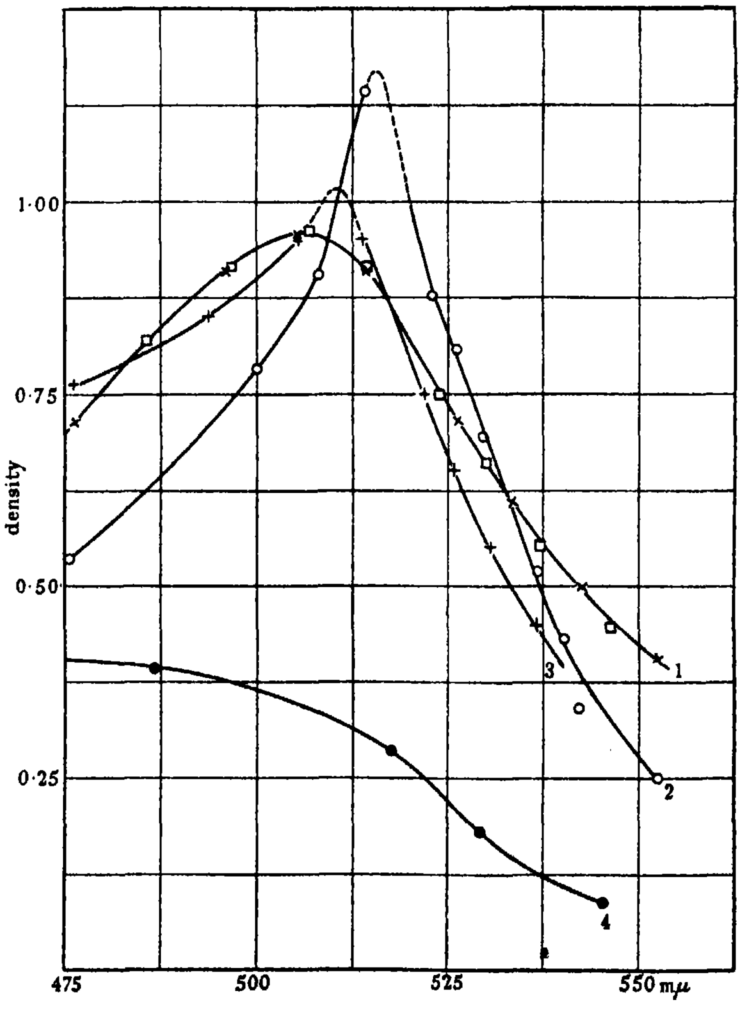

FIGURE 1. The absorption spectra of visual purple and its products (pH 6·0). Curve 1, visual purple at room temperature in water × × ×, in glycerol-water □ □ □. Curve 2, visual purple at −73°. Curve 3, primary decomposition product at −73°. Curve 4, secondary decomposition product at room temperature.

It is uncertain whether the peak of curve 2 is as smooth as shown in figure 1, as the iron spark in this region is not very rich in lines.

INTERMEDIATE PRODUCTS OF BLEACHING

The photodecomposition of visual purple at room temperature proceeds in several stages (Dartnall 1936; Lythgoe 1937; Wald 1937; Lythgoe and Quilliam 1938*a*, *b*). The primary product ('transient orange') is photostable, but undergoes a thermal change, which at room temperature is rapid enough to make its detection difficult. The spectrum of transient orange is independent of pH and the intensity of its light absorption is not much below that of visual purple (at their respective maxima). The thermal change leads to a yellow compound, the absorption spectrum of which changes with pH, and which, therefore, has been termed 'indicator yellow'. It is possible that water takes part in one or both of these reactions (Lythgoe and Quilliam 1938*a*).

Lythgoe (1937) has shown that the orange colour of a freshly bleached solution of visual purple is stable over a period of minutes, if it is kept near 0° C. The frozen retina as well as frozen solutions of visual purple are more or less photostable, and no transient orange is produced (Kühne 1879; Garten 1906; Dartnall, Goodeve and Lythgoe 1938); but, as under these conditions crystalline water must have separated from the protein, these observations have no bearing on the reaction mechanism proceeding in aqueous solution.

Procedure

To determine the intermediate reaction products at low temperature the cell was illuminated with white light by means of a 4 W lamp fixed in the optical axis of the cell at a distance of 8·5 cm. from the centre of the cell at the opposite end from the spark. The spectrum was taken before and at intervals during the illumination, with interruption of the latter. At the end of each experiment the cell was warmed up to room temperature, and the spectrum taken again.

Results

On irradiation at low temperature the spectrum smoothly changed from that of curve 2 to that of curve 3, figure 1, indicating that a chemical change had occurred. The primary product did not undergo further change after 2 hr. in the dark or on continued illumination. Figure 2 shows how the change on illumination at low temperature comes to an end. When, however, the primary product was warmed up to room temperature in the light or in the dark, it was destroyed very quickly and gave way to a secondary (final) decomposition product represented by curve 4, figure 1 and by curves 4 and 5, figure 2.

In figure 3 the absorption spectra of visual purple solutions (from different preparations) at 73° C are shown for pH 6·0 and 9·0 and also of the primary and secondary decomposition products at these two pH values. This pH

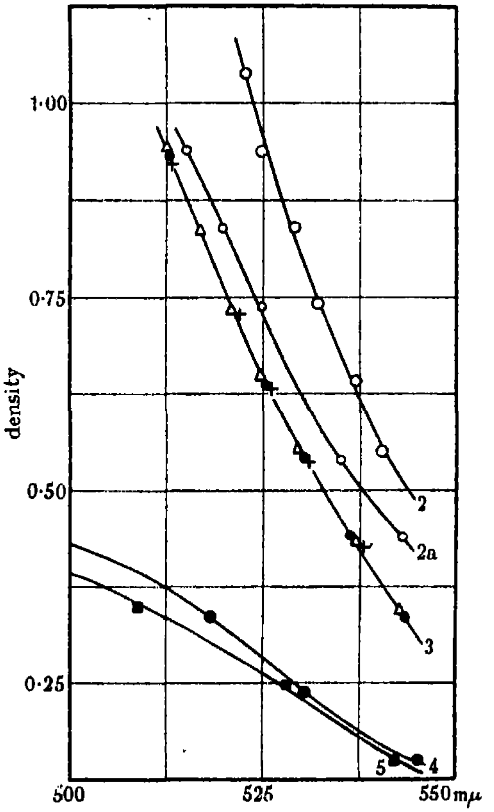

FIGURE 2

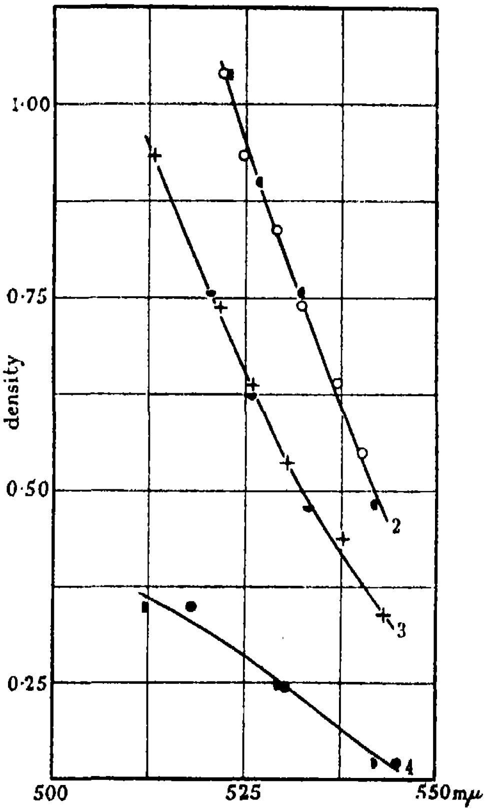

FIGURE 3

FIGURE 2. The changes of absorption in the region 510–550 mμ shown in greater detail than in figure 1. Curve 2, before illumination. Curve 2*a*, after illumination for 3 min. Curves 3, 12 min. △ △ △; 72 min. ● ● ●; and 87 min. + + +. Curve 4, after warming up to room temperature in the dark. Curve 5, 8 hr. later.

FIGURE 3. Comparison of solutions of different pH (−73°). The results for the two solutions are brought to a common basis by superimposing the curves of the unilluminated solutions at 527 mμ. Curve 2, before illumination, ○ ○ ○ pH 6·0, ◖ ◖ ◖ pH 9·0. Curve 3, after illumination, + + + pH 6·0, ▾ ▾ ▾ pH 9·0. Curve 4, after warming up, ● ● ● pH 6·0, ◗ ◗ ◗ pH 9·0.

range is the one in which the greatest effects of pH on the final product of visual purple occur (Lythgoe 1937). Results are given for the range 510–550 mμ, which is near enough to the maximum of the primary product, but where there is little influence of the 'yellow impurities' always present in visual purple solutions (Lythgoe 1937) and accurate comparison between different solutions is possible. As the concentration of visual purple solutions cannot be determined otherwise than through their optical densities, all density values of one solution had to be recalculated so that the densities of the unbleached solutions agreed at one wave-length. As will be seen from figure 3 no appreciable difference is found between the two solutions. The maximum of the absorption band of the secondary (final) reaction product (not shown in the figure) which is in any case well below 500 mμ, was however shifted to shorter waves at pH 9·0.

Discussion

A comparison of the behaviour of the primary and final reaction products of visual purple at low temperature with the corresponding products at or near room temperature, i.e. transient orange and indicator yellow respectively, shows sufficient resemblance to make it unnecessary to postulate the presence of any new species. The primary product at low temperature is photostable and has a spectrum which is independent of pH, both characteristics of transient orange. Furthermore, the maximum of its absorption curve is on the shorter wave-length side of that of visual purple (at the same temperature) and is nearly as high as the latter.

Likewise, the shape of the curve of the *final* reaction product in the low temperature work and the effect of pH on it show that this substance is indicator yellow.

The difference between the maximum at room temperature and that at -73° C is much greater with transient orange, about 30 mμ, than with visual purple, about 10 mμ; consequently, the difference in hue between illuminated and unilluminated solutions at low temperature is small and when at first a test tube containing cooled solution was exposed to daylight, and the colour examined with the naked eye, it was erroneously believed that no change at all had taken place and that visual purple is photostable at low temperature.

The stability of transient orange in cooled glycerol opens up possibilities for its closer study. It would be particularly interesting to decide whether the loosening of the chromophore-protein bond occurring on bleaching (Wald 1937) is due to the primary or to the secondary reaction, and, thus, to elucidate the nature of the transition transient orange $\rightarrow$ indicator yellow. As was first shown by Wald, indicator yellow can be extracted from its aqueous solution with organic solvents and separated from the protein. Attempts made here to extract at low temperature transient orange from glycerol-water with ethyl ether were unsuccessful. Extraction from this extremely viscous liquid, however, meets with great experimental difficulties; attempts to extract night blue from cooled glycerol-water with ether, and vice versa, were equally unsuccessful.

RATES OF REACTION

Procedure

The quantum yield of the primary reaction at low temperature was estimated by comparing the velocity of the photoreaction with the velocity in the same experimental arrangement and with the same light source at room temperature. At low temperature the progress of the reaction was measured through the percentage of primary reaction product produced. At room temperature, owing to the instability of the primary reaction product, the progress of the reaction had to be measured by determining the optical density of the illuminated solution after the dark changes had come to an end, i.e. after the transient orange was completely decomposed.

Results

It was first found that by exposing visual purple solutions in water and glycerol-water to daylight in a test tube that, at least at room temperature, both bleach at an equal rate. Although accurate measurements in glycerol-water could only be completed at low temperature (figure 2) it emerged clearly from preliminary tests that the velocity of the primary reaction is about of the same order as at room temperature. The quantum yield is not far from unity over the range 20–60° C (Dartnall, Goodeve and Lythgoe 1938) and from the above it follows that it is also of the order of unity over the wider range of temperature from − 73° C.

The secondary (thermal) reaction, on the other hand, has a considerable temperature coefficient at room temperature (Lythgoe 1937; Lythgoe and Quilliam 1938 *a*, *b*) and is immeasurably slow at − 73° C.

Our sincere thanks are due to the late Dr R. J. Lythgoe for his interest and help, to Professor A. J. Allmand, F.R.S., for his kind hospitality at King's College, London, to Miss S. J. Hooper for the preparation of visual purple, and to the Rockefeller Foundation for a financial grant.

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Address of the President Sir Henry Dale, C.B.E., at the Anniversary Meeting, 1 December 1941

This is the first occasion on which I have had the great honour of addressing the Royal Society on this anniversary of its foundation. According to custom, I begin with brief mention of those whom death has taken from our Fellowship during the past year, and whose memories we honour.

ALFRED YOUNG (1873–1940), distinguished for his contributions to pure mathematics, was half brother to another of our Fellows, Sydney Young, a chemist of eminence. Alfred Young had an insight into the symbolic structure and manipulation of algebra, which gave him a special place among his mathematical contemporaries. After a successful career at Cambridge he entered the Church, and passed his later years in the country rectory of Birdbrook, Essex. His devotion to mathematics continued, however, throughout his life, and he published a steady stream of work in the branch of algebra which he had invented, and named 'quantitative substitutional analysis'. He lived to see his methods adopted by Weyl in his quantum mechanics and spectroscopy. He was elected to our Fellowship in 1934.

With the death of MILES WALKER (1868–1941) the Society loses a pioneer in large-scale electrical engineering. Walker was a man of wide interests. He was trained first for the law, and even followed its practice for a period. Later he studied electrical engineering under Sylvanus Thompson at the Finsbury Technical College and became his assistant for several years. Thereafter, encouraged by Thompson, he entered St John's College, Cambridge, with a scholarship, and graduated with 1st Class Honours in both the Natural Sciences and the Engineering Tripos.

Having entered the service of the British Westinghouse Company, he was sent by them to the United States of America to study electrical engineering with the parent company in Pittsburgh. On his return to England he became their leading designer of high-speed electrical generators.

In 1911 Walker became, for a year, lecturer in turbo-machinery and rotary-converter design at the Imperial College of Science and Technology, and then, in 1912, was appointed Professor of Electrical Engineering at the Manchester College of Technology. There he instilled into a generation of Manchester electrical engineers the importance of going back to fundamental principles in all discussions of applied problems.

Throughout his life Walker retained an enthusiastic interest in research, and this, together with his enquiring and inventive mind, made him a real source of inspiration to his students. He was elected to our Fellowship in 1931.

HENRY SELBY HELE-SHAW (1854–1941) achieved distinction in scientific research and invention, and also as a pioneer of Technical Education.

After a brilliantly successful career as a student, he became the first Professor of Engineering in Bristol University at the early age of 27. Later he founded two other Schools of Engineering, becoming the first Professor in that faculty at Liverpool University, and first Professor and subsequently Principal of the Transvaal Technical Institution. His last, and perhaps his greatest contribution to technical education is to be found in the part which he played in founding the scheme of 'National Certificates', of which he was joint chairman until 1937. His scientific researches covered several fields, but his most distinctive contribution lay in the development of his stream-line flow methods, which did much to bring hydrodynamics within the range of the experimental sciences. It may be, however, that Hele-Shaw's most lasting fame will be due to his achievements as an inventor. From his intense and active interest in the practical and scientific development of the motor car came his friction clutch and his system of hydraulic transmission. From his researches on stream-line flow came his stream-line filter. From his equally active interest in the development of the aeroplane came his Automatic Variable-Pitch Propeller and his Exactor Control.

Hele-Shaw took a prominent place in the development of the organized professional life of engineers, becoming president of several engineering societies, including the Institution of Mechanical Engineers.

He became a Fellow of our Society in 1899.

CRESSWELL SHEARER (1874–1941) was an interesting personality whose main scientific activities were in experimental embryology. He spent much time at Naples when the Zoological Station there was in, perhaps, its most famous period. He settled in Cambridge in 1910, and shortly afterwards was appointed University Lecturer in Experimental Zoology, being the first to hold that appointment. The ensuing decade was a period of active and productive research for Shearer, and of inspiration to his students. He published important papers on the development of trochosphere larvae and on sex determination in *Dinophilus*; and in 1913 came the well-known monograph in our *Philosophical Transactions*, in collaboration with de Morgan and Munro Fox, on 'The experimental hybridization of echinoids'. Shearer was elected to our Fellowship in 1916, his activities being at that time diverted by the national emergency to war-time bacteriology. Later he carried out important researches on the metabolism of the eggs of sea urchins, being the first to demonstrate the remarkable changes in oxygen consumption which occur for a few seconds, after the fertilizing spermatozoon has entered the egg. Apart from his scientific interests, Shearer was a man of wide culture, and in 1935 published a book on *The renaissance of architecture in southern Italy*.

ARTHUR THOMAS MASTERMAN (1869–1941) studied and graduated at Cambridge, and was early appointed Assistant Professor of Natural History at St Andrews under the late Professor W. C. McIntosh. Under McIntosh's influence he took up the study of the life history of the British food fishes, which for a quarter of a century remained the chief subject of his researches. He became known also for work in other fields of marine zoology, on *Phoronis*, *Cephalodiscus* and *Cribrella*. In 1903 he accepted appointment as a scientific expert attached to the English Ministry of Fisheries, and did important work in reorganizing the statistical records of the Department. On retiring from that post he became interested in a proprietary hypochlorite preparation, and published shortly before his death some investigations made in that connexion.

The death of WILLIAM BULLOCH (1868–1941) has removed a central and familiar figure from the circle of British pathologists and bacteriologists. After a distinguished academic career in medicine at Aberdeen, he went for post-graduate study to Leipzig and other continental centres, forming and retaining an unusually wide circle of friendships and scientific contacts among the pathologists of Europe. Returning to this country, and having worked for a time under Ferrier and Horsley, be became Bacteriologist to the Serum Laboratories of the British Institute (now the Lister Institute) of Preventive Medicine, and later Bacteriologist to the London Hospital, and finally Professor of that subject in its Medical School. Bulloch had a knowledge of his subject unrivalled in its width and detailed accuracy, and he was a consistent and methodical worker; but, though he published observations over a wide range, the more significant dealing with immunology and haemolytic action, he early became more interested in the history of his subject, and in the critical analysis of its records, than in experimental additions to it on his own account. He had inherited a flair for bibliographic enquiries, and he became increasingly concerned with the historical setting of any subject he touched, and with the independent verification of every statement from the original authorities. His critical examination of the recorded pedigrees of familial diseases, for the *Treasury of human inheritance*, ranked him as a pioneer in human genetics; while his *History of bacteriology*, published in 1938, at once attained general recognition as a masterpiece. This Society itself owes an immense debt to Bulloch's scholarly devotion to the history of science and his desire for accurate personal records of those concerned in its advancement. His *Roll of the Fellows of the Royal Society*, embodying the results of many years of unremitting labour, has now been acquired by the Society by friendly arrangement with his widow.

Bulloch was a man of strong and remarkable personality, with an unrivalled store of personal memories of the great figures in the medical science of his time, and had served on a wide range of expert advisory boards and committees.

The name of Sir FREDERICK GRANT BANTING (1891–1941) will always, and rightly, be associated with the discovery of insulin, and the dramatically sudden change which it produced in the accessibility of a distressing disease to treatment. Banting had seen active service and had been seriously wounded in France in 1915, before he was sent back to Toronto to finish his medical course. After the war he went into practice in London, Ontario, holding also a part-time teaching post in physiology at the university. It was there, in 1921, that the vision came to him, and sent him back to Toronto to beg the late Professor Macleod for research facilities in the Department of Physiology. The discovery which resulted from the determined attack by Banting and a still younger collaborator, C. H. Best, on a problem which had baffled so many workers of ripe experience, provided a romantic chapter in the story of modern researches. Banting became a centre of popular interest, and his proud and grateful country provided him with an Institute of Medical Research. It was not to be expected that Banting would produce another discovery to rank with that of insulin in appeal to the world's imagination. Important results, however, were soon coming in steady output from his laboratories; their work on silicosis, a disease of great importance in Canada's mining industry, deserves particular mention. With the outbreak of another war, Banting became a natural leader in the application of Canada's medical researches to military problems, and he threw himself into the work with tremendous energy and enthusiasm. Death came to him in his fiftieth year, through failure of an aircraft which was bringing him on an urgent mission to England. It is idle to speculate as to what he might yet have done for science. His name is for ever linked to a discovery great in itself, and even greater in its influence on subsequent medical researches. He became a Fellow of the Society in 1935.

JOHN SMYTH MACDONALD (1867–1941), after graduating in natural science at Cambridge, completed his medical studies at Liverpool, where his interest in physiology was stimulated by contact with Sir Charles Sherrington. After holding minor appointments in other universities, he became Professor of Physiology at Sheffield, and later, in 1914, succeeded Sir Charles Sherrington at Liverpool. His earlier researches dealt with the relations between electrolytes and colloids in nerve and muscle fibres, and particularly with the localization therein of potassium and chlorides. On these he based conceptions of the electromotive phenomena in these structures, and of muscular contraction. His later work dealt with the production of heat and the output of energy by man during exercise, for the study of which he built a large calorimeter chamber at Sheffield.

HERBERT MAX FREUNDLICH (1880–1941), elected a Foreign Member of the Society in 1939, was born at Charlottenburg in 1880 of a German father and a Scottish mother. A pupil of Wilhelm Ostwald, he worked after graduation for eight years as assistant in Ostwald's Institute. There followed five years as Professor of Physical Chemistry in Braunschweig, and another seventeen as head of a department in Haber's Institute at Dahlem. The revolution in Germany in 1933 brought him to London, as an honorary Research Associate in the Chemistry Department at University College, with support from Imperial Chemical Industries; and in 1938 he went as Distinguished Service Professor of Colloidal Chemistry to the University of Minnesota, Minneapolis, where he died last March. Any approach, even, to detailed mention of Freundlich's contributions to the fundamental physical chemistry of colloidal systems is beyond the scope of this occasion. It must suffice to say that his researches and those of his pupils and collaborators covered an immense field. He created one of the most important schools of research in the chemistry and physics of colloids, and by his books, especially the successive editions of his famous *Kapillarchemie*, as well as by his researches, he became one of the chief founders of the modern science of colloids.

ARTHUR LAPWORTH (1872–1941) was the son of the distinguished first Professor of Geology at Birmingham University.

From 1913 to 1922 he occupied the Chair of Organic Chemistry in the University of Manchester, and from 1922 to 1935 he was Sir Samuel Hall Professor of Chemistry and Director of the Laboratories. He was elected a Fellow of the Society in 1913 and was awarded the Davy Medal in 1931.

Lapworth's scientific work was characterized by remarkable insight and breadth of outlook. His interest was chiefly concentrated on the intimate mechanism of chemical change, and for many years his views were so advanced that his contemporaries paid little attention to them. Now they are the commonplaces of the text-books, and we are able to appreciate what a great part Lapworth played in guiding the course of development of electrochemical theories of organic chemistry. The prophet was also a superb experimentalist, and some of the topics he illuminated were the following: migrations, cyanohydrin formation and decomposition, the constitution of camphor and gingerol, esterification and acid-base catalysis in general, the mechanism of the benzoin reaction, formation and properties of sulphonic acids and their esters, mechanism of bromination of acetone, and many miscellaneous subjects in organic and physical chemistry. Above all he will be remembered for his theoretical papers and his brilliant ideas on the classification of anionoid and cationoid reagents.

By the sudden death of Sir ALBERT SEWARD (1863–1941) we have lost a colleague who had won a position of high distinction and authority in his own departments of science, and who had given generously of his time and his interest to the work of the Society and the promotion of its interests. At the University of Cambridge he was University Lecturer in Botany from 1890 to 1906, Professor of Botany for the ensuing 30 years, and Master of Downing College, in addition, for the last 21 of these. He served his term as Vice-Chancellor, and was an Honorary Fellow of St John's, Emmanuel and Downing Colleges. The progress of scientific studies, and especially of botany, at Cambridge owes much, indeed, to his ability as a teacher and an administrator, and many scientific bodies and institutions were indebted to his wisdom and his energy. His own many and varied contributions to knowledge by research dealt with fossil plants and gave him a leading position among palaeo-botanists. Our Society recognized their distinguished merit by the award of the Royal and the Darwin Medals, and they gave him high standing in geology as in botany. He had been President of the Geological Society, as well as of the Botanical Section of the British Association and of the International Botanical Congress. He worked hard for international co-operation in science and for its re-establishment after the last war. With his friend, the late Professor Went of Utrecht, he was largely responsible for the initiative which led to the formation of the International Union of Biological Sciences, of which he became the President. There was a special fitness therefore in Seward's election in 1934 as the Royal Society's Foreign Secretary. He had already served for two earlier periods on our Council. When he retired from his Cambridge Chair and College Mastership, in 1936, he settled in London, and was able to give even more fully of his time and attention to the affairs of the Society and the maintenance of its foreign connexions. His ripe experience, his wisdom and the charm of his distinguished personality will be sadly missed by a wide circle of men of science in many countries.

George William Clarkson Kaye (1880–1941) had been since 1922 the Superintendent of the Department of Physics at the National Physical Laboratory. During the earlier part of the war of 1914–18 he had served with distinction in the Royal Engineers and the Royal Air Force, and from 1917 to 1920 he held the post of Chief Inspector of Materials to the Air Ministry. Kaye's public-spirited activities earned for him the gratitude of students and workers in several departments of science. Physicists have had occasion to be grateful for his work as co-author of the book of *Tables of physical and chemical constants*. Those concerned with the use of X-rays and radium for medical and other purposes are deeply indebted to his work on the dangers of repeated exposure and the means of protection against them. He had been President of the Röntgen Society, had acted as honorary editor of journals dealing with radiology and radiography, as Secretary of the International X-ray and Radium Protection Committee, and as Secretary of the National Radium Commission. He devoted much time and thought to the planning of the New Physics Building at the National Physical Laboratory. His greatest pride was in the Acoustics Laboratory, specially designed for the experimental study of the acoustics of buildings; and he was active in the quantitative study of the problem of noise in our modern civilization, with a view to means for its elimination. By his work as a scientific administrator and organizer, indeed, both national and international, Kaye did even greater service to science and to his fellow men than by his own personal work as an investigator. He was elected to our Fellowship in 1939.

Among classical scholars Sir James Frazer (1854–1941) was a pioneer in the study of the evolution of classical civilization from the rudimentary notions of lowly cultivators, applying the comparative method which his friend and guide, Robertson Smith, had begun to use in his studies of Hebrew Religion. Both Smith and Frazer acknowledged their debt to J. F. M'Lennan. Frazer developed his life-long studies of the evolution of religious ideas and practices into the twelve volumes of *The Golden Bough* and a vast number of accessory works, which have been listed by Besterman in the *Bibliography of Sir James George Frazer* (Macmillan, 1934). In addition to being a Fellow of this Society he was a member of the Order of Merit, a foundation Fellow of the British Academy, an Associate Member of the Institut de France and the recipient of almost innumerable honours from learned societies and universities, honours which seemed not to touch his modest shyness or his care for every detail. He thought that his theories would be overlaid as better data were collected by field workers, and as the divergent adaptations of society to many environments came to be better understood; but he felt that man's thoughts, beliefs and practices were products of natural evolution, and his splendidly phrased expositions impressed that feeling upon a wide public. His was one of the outstanding influences on the thought of our time. He was elected to the Fellowship in 1920.

The name of Lord CADMAN (1877–1941) will always be associated with petroleum, for not only did he found the first school in the Empire of petroleum technology, but he became also head of two of the greatest petroleum companies in the world. For thirteen years he was Professor of Mining in the University of Birmingham and collaborated with the late Professor J. S. Haldane in a fruitful series of investigations into mining hazards.

As a leader of industry he won the admiration of men of science by his unceasing advocacy of the application of science to industry. It has been stated he never looked at to-morrow, but the day after to-morrow, for he looked upon scientific research as shaping the destinies of industry in generations to come.

The scientific development of the oil field in Iran will always be associated with his name, for though his academic life of thirteen years was that of a Professor of Mining, he was extremely well versed in all aspects of geophysical exploration. There was probably no man of his time with the same wide and encyclopaedic knowledge of the oil industry. He was elected a Fellow in 1940.

ROBERT ROBISON (1883–1941) was educated at University College, Nottingham, and at the University of Leipzig. Almost the whole of his scientific career was spent at the Lister Institute, to the staff of which he was appointed in 1913 and where he succeeded the late Sir Arthur Harden as Head of the Department of Biochemistry in 1931.

Robison will be remembered as one of the outstanding British biochemists of the last 20 years, and his name will be particularly associated with two important discoveries. Following up the work of Harden and Young, he isolated from the products of fermentation of sugar by yeast a hexosemonophosphate; this substance, which is now commonly referred to in the scientific literature as the 'Robison ester', has proved to be of great significance in intermediary carbohydrate metabolism. Robison's second important achievement was the demonstration in ossifying cartilage of an enzyme which hydrolyses hexosephosphates; this discovery was followed by a long series of papers on the role of the phosphatase in the process of ossification.

Apart from his personal achievements Robison contributed greatly to the advance of biochemistry by the services which he gave to scientific bodies outside his own Institute and by the example of a life devoted to the highest standards of research. He was elected a Fellow in 1930 and served on the Council from 1934 to 1936.

Sir ARTHUR JOHN EVANS (1851–1941), who died on 11 July, three days after his ninetieth birthday, retained to the last his wide archaeological interests and critical appreciation of ancient craftsmanship. After a precocious childhood guided by his distinguished father, Sir John Evans, a Fellow of this Society and its Treasurer, he spent some years in adventurous travel in Balkan lands, and then became Keeper of the Ashmolean Museum at Oxford in 1882 and transformed it into an active centre of archaeological research. His special interest in engraved gems led him in 1894 to the discovery, on Cretan seal-stones, of a pre-Phoenician system of pictographic and linear writing; and this in turn led to his acquisition and excavation of the site of the prehistoric palace at Knossos, with frescoes and other works of art, and many clay tablets inscribed in the new form of writing. Ably supported throughout by devoted helpers, he retained nevertheless in his own hands the voluminous publication of this vast mass of material, which his wide experience and imaginative insight enabled him to interpret as it deserved. Far beyond his scientific eminence, he will be remembered for the charm and simplicity of his character, his generous use of ample resources, his devotion to his many friends, his unselfish encouragement of colleagues and younger students, and his vigorous defence of national, academic, and intellectual freedom. He was elected a Fellow in 1901 and received the Copley Medal in 1936.

With the death of **Alfred Joseph Clark** (1885–1941), at the early age of 56 years, British pharmacology loses a valued leader, and medical science a stimulating influence and a wise counsellor. After study and graduation at Cambridge and St Bartholomew's Hospital, Clark held Chairs of Pharmacology at Cape Town, University College, London, and finally Edinburgh, being successor to his former chief, A. R. Cushny, in the two last of these. Clark's own researches showed him as concerned primarily with the fundamental physiology of contractile tissues, and with the actions of drugs, mainly for the light which they could throw on these problems. At the same time he had a keen interest in practical medicine, and became an effective and stimulating teacher on the action of drugs as the basis of their rational use in treatment. These two separate streams of interest found expression in his books. He considered the more fundamental problems in books and monographs on *The mode of action of drugs on cells*, *Comparative physiology of the heart*, *Metabolism of the frog's heart*, and *General pharmacology*, and he dealt with practical applications in a stimulating text-book of *Applied pharmacology*. The more practical aspects of his ability came to the front also in the two great wars of our time. In that of 1914–18 he served with distinction as a medical officer in France, being awarded a Military Cross and attaining the rank of Major. In the present war, being on a special mission of medical and scientific liaison with the British Army in France, he took part in the retreat, and did fine emergency service as a regimental medical officer. Clark became a Fellow of the Society in 1931. His death takes all too early from medical science a worker whose imaginative mind, devotion to research and service to human need have exerted a deep and far-reaching influence on its progress.

James Charles Philip (1873–1941), after graduation in chemistry at Aberdeen, went for special experience of physical chemistry to Nernst, in Göttingen. On his return to this country he worked under H. E. Armstrong at South Kensington and with Heycock and Neville in Cambridge. His career from 1900 onwards was closely associated with the Royal College of Science, South Kensington, before and after its incorporation in the Imperial College. He became Assistant Professor in 1909, and the first Professor of Physical Chemistry in 1913, succeeding H. B. Baker as director of the Laboratories of Inorganic and Physical Chemistry in 1932. Philip, until the most recent years, was actively engaged in researches, into which he drew a succession of students in collaboration, covering a wide range of subjects in physical chemistry; and he has left behind him an important and active centre for that subject. But apart from these distinguished personal activities in science, it was characteristic of Philip that for many years he accepted without question, and discharged with quiet efficiency, a load of duties on behalf of his colleagues, of his university and of the nation, such as might well have filled the whole time and interest of a man less unselfish and devoted. Already during the war of 1914–18 he was acting as Secretary of the Royal Society's Committee which organized the services of the nation's chemists, especially for the production of certain needed remedies. In later years he served on the Senate of London University, was dean of the Faculty of Science in 1934 and deputy Vice-Chancellor in 1937–38. Still more recently he was Chairman of the Committee of the Royal Society and the Ministry of Labour dealing with the section of the Central Register concerned with chemists, and accepted, in addition, a long and exacting term of duty, demanding firm friendliness and understanding, as Chairman of the University of London Joint Recruiting Board. He was President of the Society of Chemical Industry from 1939 to 1941, while the Chemical Society, to which he had acted as Secretary for eleven years, and Chairman of its Bureau of Abstracts for nine years, elected him to its Presidential Chair a few months before his sudden death. Though he retired from his Chair at the Imperial College in 1938, he returned to arduous duty as Deputy Rector, when Sir Henry Tizard's services were claimed elsewhere. Seldom has a life been more fully devoted to science, to the public service, and to the interests of colleagues, friends and pupils. Philip was elected to our Fellowship in 1921, and served on our Council from 1928 to 1930.

Paul Sabatier (1854–1941), who was elected to our Foreign Membership in 1918, began his researches into problems of catalysis in 1897, and in 1900 entered upon his studies of catalytic hydrogenation, which have had such a great influence on the progress of organic chemistry, in research and in industry, and for which he was awarded the Nobel Prize in Chemistry in 1912. Among Sabatier's numerous contributions to this aspect of chemistry may be mentioned his discovery that the same catalyst, by appropriately varying the conditions, can be made effective for either hydrogenation or dehydrogenation, and his studies of selective catalysis, with its suggestive bearing on biological processes. He was led to formulate a theory of catalytic action, involving the formation of temporary combinations, which is similar in essence to that which still prevails. It is sad that the news of his death at Toulouse should come to us under present conditions.

WALTER GARDINER (1859–1941). At the time of his election, in 1890, Walter Gardiner was the youngest of our Fellows, being admitted at the early age of 31 years. At the time of his death this year he was one of the veterans, being at the end of his 82nd year. His period of intense scientific activity, however, establishing him as one of the eminent botanists of his time, extended only from 1881 to 1907. The work which brought him fame was concerned with the histological demonstration of the continuity of the cells of vegetable tissues, due to connecting fibrils of protoplasm, the regular presence of which, *ab initio*, he was the first to reveal. He began this work under Sachs at Würzburg in 1882, and continued it mainly in his own private laboratory in Cambridge and the Jodrell Laboratory at Kew. The Royal Society recognized its importance in recommending him for a Royal Medal in 1898. His activities in research, so far as they led to published record, came early to an end, however, in 1907, the later part of his life being clouded by ill-health, so that he was personally unknown to the botanists of the younger generation. This enforced withdrawal from activity, of an investigator of such unusual powers, was a great loss to the science of botany.

ARTHUR GEORGE GREEN (1864–1941) was one of the veterans of organic chemistry, particularly in its application to the synthetic manufacture of dyestuffs in this country. After training and graduation at University College, London, he went, in succession to Meldola, as research chemist to the firm of Brooke, Simpson and Spiller, which had succeeded to the industrial interests of W. H. Perkin. In nine years with this firm and a further seven with the Clayton Aniline Company, Green made a number of important improvements in artificial dyestuffs and their applications. In 1903 he accepted the Chair of Colour Chemistry and Dyeing at the University of Leeds, and, in the next eleven years, earned a high reputation for that school as a centre of progressive research and teaching in its subject. He returned to work directly for industry in 1916, when he became Director of Research to the British Dyestuffs Corporation, then faced with the task of re-establishing in this country, during war, an industry which had been allowed to pass so predominantly into German hands. In 1923 Green established himself in London as a consultant to a number of firms, both British and American, engaged in chemical manufacture. In this connexion he retained to an unusually advanced age his practical and research activities in the science to which he had given effective service for so many years. He was elected to our Fellowship in 1915.

John Stanley Plaskett (1865–1941) died on 17 October at Victoria, British Columbia, where he had been in charge of the Dominion Astrophysical Observatory from 1917 to 1935. His original work on the application of spectroscopy and the design of spectroscopes for the determination of stellar radial velocities, and on the velocities, distances and properties of the O-type stars, had brought him international recognition, and the honour of having his name attached to the massive bright star, the 'Plaskett Star', which he discovered.

Plaskett's formal, academic education came to him unusually late in life, and he had served an apprenticeship and held a post with the Edison Companies of the United States and Canada before he entered Toronto University and eventually graduated in mathematics and physics. Researches in photography and stereoscopy brought him an astronomical appointment with the Dominion Government, and his successful leadership of an Eclipse Expedition to Labrador was followed by his appointment as astronomer to the Ottawa Observatory. Here he was able to begin his important series of spectroscopic determinations, but the inadequacy of the 15-inch telescope for the purpose led him, with the consent of the Government, to raise funds sufficient for the construction of a 72-inch instrument. This was installed at Victoria, B.C., where Plaskett took charge of it and spent the remainder of his working life.

Many honours and international recognitions came to him, and he was elected to our Fellowship in 1923.

Edgar Vincent, Viscount D'Abernon (1857–1941), elected under Statute 12 in 1934, was a Fellow of the Society of a type much more frequent in its earlier history than in recent years. One can imagine him, indeed, well in the picture among the distinguished group of our founders. A brilliant figure in Society, a connoisseur of the arts and an enthusiastic follower of more than one sport, an able man of affairs and a distinguished public servant, he became a convinced admirer and advocate of science, with a strong sense of the need of its guidance in policy. It was not, however, till relatively late in his career that he made any close scientific contacts. He accepted in 1915 the chairmanship of the Central Control Board (Liquor Traffic) at its war-time foundation, and experience of conflicting propaganda soon determined him to seek a firmer basis for practical action, in independent and strictly scientific evidence concerning the action of alcohol on man. He accordingly appointed a Scientific Advisory Committee to draw up an impartial account of existing knowledge and to plan researches to fill any gaps so revealed. The members of this Committee were Fellows of this Society, and two of them have since occupied this Presidential Chair. Their report, at Lord D'Abernon's request, was cast in a form suitable for general reading, and he himself contributed an able and constructive preface. He found time to pay frequent visits to those who undertook the researches, and even to witness experiments. Some years later, on returning from his historic mission as the first British Ambassador to Berlin after peace was signed, he presented an able statistical report on post-war economic conditions in Germany, and was elected President of the Royal Statistical Society. Later, again, he did service of great importance to different fields and aspects of science, as Chairman of the Royal Commission on Public Museums and Galleries, and finally as Chairman of the Medical Research Council, till the long illness which clouded the remaining years obliged him to withdraw from all activities.

The news of Sir ARTHUR HILL'S (1875–1941) sudden death from a riding accident has come as a shock to a wide circle of friends. Only a few weeks ago the Council had asked him to undertake the obituary notice of Walter Gardiner. It was characteristic of Hill's devotion to the memory of the old teacher who had guided his first steps in research, and of his quiet efficiency in discharging a duty of friendship, that the notice of Gardiner was already written, corrected and lying in page proof in our office, when the tragic mishap took Hill from us.

From Cambridge where his career as a botanist began under Marshall Ward and Gardiner, and where he published important papers on botanical histology, dealing with sieve tubes and continuity of protoplasm between cells, Hill went to Kew in 1907 as assistant director under Sir David Prain. He remained in that position for fifteen years, during which he was able to continue his researches. Though primarily a morphologist, he was interested in plants in all their aspects, and published a number of papers on the taxonomy of different genera. Later he travelled widely with the aid of the Empire Marketing Board, interesting himself in the economic applications of botany as well as in making new contacts for Kew, by which its collections became enriched and its services rendered more widely available. As director at Kew he encouraged travel by his staff to make direct acquaintance with Empire flora, and obtained grants to make these journeys possible. He became, indeed, an effective ambassador for botany in the British Empire. In other circles also Hill was well known and his counsel prized. He kept a warm affection for his old school, Marlborough, and interest in its affairs, and he was an active and valued member of the

University Grants Committee. He served with distinction in the war of 1914–18, and was detailed for special duty as horticultural adviser to the War Graves Commission. He was elected a Fellow in 1920.

WALTER NERNST (1864–1941), who was elected to our Foreign Membership in 1932, was a great figure of the period, early in the present century, in which the modern science of physical chemistry was taking shape. He was awarded a Nobel Prize for Physics in 1920. To him was due much of the fundamental work on the reversible galvanic cell and, in relation to the theory of solutions, on diffusion and partition, and on the hydration and dissociation of electrolytes. In 1906 he formulated a thermodynamical theorem by which the free energy could be determined from the known heat of a reaction. This theory, in its full development, found application in the realm of the absolute zero on the one hand, and, on the other, at such high temperatures as exist only in the stars. Nernst was thus led to the discovery that the specific heat of an element vanishes at a sufficiently low temperature—a conclusion which, in its harmony with Einstein's application of Planck's work, provided one of the surest experimental foundations for the quantum theory.

Apart from his own researches, Nernst was one of the great teachers of his time, whose personality and inspiration meant much for science through the pupils and research students who came from all the world to his Institute in Göttingen, and, later, in Berlin.

Nernst's contacts with many of those, who were thus bound to him by ties of friendship and grateful esteem, were twice broken by war. When the present war loomed imminent in July 1939, he wrote thus to one of our Fellows: 'Jedenfalls bin ich sicher dass unsere Freundschaft sich als unzerstörbar sich erweisen wird, was auch kommen mag.' ('In any case I am certain that our friendship will prove to be indestructible, whatever may come.') Nothing that has come will deter us from paying our tribute of respect to the memory of the great man of science, whom death has now taken from our Foreign Membership.

News of the death of Dr HARRY ELTRINGHAM (1873–1941) comes too recently to allow more than the briefest mention, on this occasion, of his career and his services to science. The son of a shipbuilder, and himself for some time a partner in the family business, he was one of a number of distinguished amateur naturalists who have been elected to our Fellowship, and whose work has made a contribution to the advancement of scientific knowledge which is, perhaps, especially characteristic of British science. Eltringham became a recognized leader in Entomology, serving as Publications Secretary, Vice-President, and President of the Royal Entomological Society of London. He gave to protective mimicry a special preeminence in his studies, and did pioneer work on the minute structure of the sense organs in the Lepidoptera. For some years before he retired to the country he lived in Oxford, carrying out his observations and experiments in connexion with the University Museum. He was elected to our Fellowship in 1930.

Only to-day comes the news of the death, last Friday, of WALCOT GIBSON (1865–1941), who, after geological work in South and East Africa in the early nineties of last century, joined the staff of H.M. Geological Survey and became director of the Survey for Scotland. He was elected to our Fellowship in 1925.

I have made mention of the late Professor Bulloch's very important contribution to our history, in his detailed record of the career and the claims to distinction, so far as these were discoverable, of every one of our Fellows, from the beginning of the Society almost to the present day. Concerning some, whose elections duly appear in our records, no additional information was obtainable, even by Professor Bulloch's researches. It is unlikely that any of these were important contributors to science; the Society in its earlier days, and even up to 1847, having admitted a substantial proportion of Fellows whose interest in its scientific activities was not more than that of onlookers. An interesting article by Sir Henry Lyons, in the recent issue of *Notes and Records*, shows that this was true even of many of the Society's earlier officers. On the other hand, the history of the doings of our Fellows would, over a long period, have comprised practically the whole of the history of science in this country. The failure to discover anything at all about some of them gives new prominence to the lack in our organization, from the beginning of the Society until now, of a system for collecting, during the lives of our Fellows, any but the most meagre items of information concerning them and their doings. This defect is further illustrated by a recent acquisition of papers which concern one of the most important figures in the history of the Society—Sir Robert Moray, who was actually its first President, before the Society received its first Charter, which Moray's influence with King Charles II appears, indeed, to have been largely instrumental in obtaining. Little else was known of the man who played so prominent a part in our foundation, till a life of him was written by the late Alexander Robertson of Edinburgh, though this was not published till after the death of the author in the war of 1914–18. Apart from our earliest Journal Book and certain letters preserved in our library, Robertson obtained his material mainly from the Kincardine papers—a long series of letters from Moray to the second Earl of Kincardine. The originals of these have been lost, and it is not known whether they still exist. Robertson had access to a manuscript copy, but this also disappeared after he had used it. The recent rediscovery of this unique copy is due to the watchful interest of one of our Fellows, Mr Clifford Dobell, who has acquired it and generously offered it to our library, which will henceforward be practically complete in documentary sources for all that is known concerning our first President. In offering the Society's thanks to Mr Dobell for this, not the only example of his generous interest in our library and our history, I venture to appeal to others of our Fellows who are learned in such matters, for help in bringing to the Society's notice opportunities of adding to its treasury of documents bearing on the history of the Society and of science.

So much for the records of the past. A few weeks ago the Fellows received a communication from me, introducing, with the Council's approval, a new scheme which should make good this gap in our equipment, so far as the future is concerned. If we can make this scheme a success, it should provide the Society with a complete set of personal records of all the Fellows from now onwards, containing all the relevant facts concerning the ancestry, parentage and education of each, and a full account of his scientific opportunities, experiences and achievements. For those who now and in the future are newly elected to our Fellowship, it should be a matter of small trouble to provide such accounts of their careers up to the time of election, and then to keep them up to date by annual addenda. It will certainly involve a greater initial trouble for those of us who have become seniors in the Fellowship, in the absence of any system of this kind. On the other hand, it seems to me almost a commonplace to suggest that the memories of some who are still with us cover a period in which the change and the expansion of scientific knowledge have been so rapid and so revolutionary, that the future, looking back upon these times, is likely to regard them as unsurpassed in interest and importance, in the whole history of science. In his Address from this Chair a year ago, Sir William Bragg, speaking of J. J. Thomson and Oliver Lodge, who had passed from us during the year then ending, referred to the memory, still preserved by himself and by others of his own seniority, of 'the brilliant years when these men and their contemporaries were writing the...first pages' of a great new chapter of science. I am appealing to those who keep such memories to put them on record now, and to commit them to the keeping of the Society, for those who will come after them here, and for all who will be interested in tracing and interpreting the history of science over the great epoch of the past half century. No doubt we shall soon have full-scale biographies of men like Thomson and Lodge, in which the attempt will be made to recover, from such correspondence and notes as may be available, their impressions and memories, in turn, of the great men of the generation which went before them. How much, one thinks, might the task of the biographers have been eased, and how much might have been kept for us which no diligence can now recover, if all the leading men of science of that time had been making for the Society records of the kind which we are now trying to inaugurate. To mention another department of science, do we not wish that we had had such a system in use when some of our Fellows could have left us notes of work and discussion with Claude Bernard, Louis Pasteur, Robert Koch, Carl Ludwig and others of that great period of the latter half of the 19th century?

And there are memories of a later generation which we must hope to get now on record, and to hand on to our successors. To quote only one example, there are still a number of us who remember Ernest Rutherford as he first came to Cambridge, as a post-graduate research student from New Zealand, and many more who were later his direct and intimate pupils and collaborators. I am sure, also, that Sir William Bragg was right, when he told us last year that 'our young men of to-day are maintaining in full force the tradition that they have received'. The interval between the two wars showed us how quickly science can return to its normal direction of progress, at some points apparently with even an added vigour, after the forced diversion. I have no doubt at all, that, when we can free ourselves again from such duties as now preoccupy brain and hand for most of us, we shall find that we are still in the great period, and that science will rapidly regain the momentum of its advance. Experience suggests that we may find that it has even, in some directions, made unexpected and almost unnoticed leaps forward. I make a strong appeal, then, to the Fellows also of more recent election to take promptly what, for them, will be the relatively small trouble of rendering and maintaining the accounts of their doings and experiences in science, for the Society's new files of personal records. The present century has witnessed a prodigious growth of the number of those who, in one capacity or another, are directly concerned with the advancement of natural knowledge by research. The election to our Fellowship has, of necessity, become more closely selective and exclusive, and we can hardly expect to avoid a few mistakes of choice in either direction. Nevertheless, I think that we can assume, without undue self-complacency, that a detailed and systematic record, of the scientific activities and contacts of all the Fellows of this Society, will continue to provide a fair picture of the main currents, at least, of scientific progress in this country. Those who in the future endeavour to write the general history of our period may well find in the outburst of scientific achievement its distinguishing and central character, far transcending in importance all changes of political structure. It is not for us, indeed, to anticipate such judgements, but I think that it is part of our duty and concern to see that the material for them is provided. If our Fellows will make the small, systematic effort needed to ensure the success of the new scheme, the future historian of the science of our times, unlike those who now laboriously seek and interpret the scattered relics of its earlier days, should find ready to hand an invaluable body of authentic and contemporary data.

As we come to the end of another year we can see, as yet, no prospect for science of escape from urgent preoccupation with the means of waging war. On the contrary, with the Union of Soviet Russia now locked in a supreme struggle for its own existence and the world's freedom, and with the United States of America rapidly directing its tremendous scientific and technical potential to the support of the same great cause, the diversion of science from its normal uses and objectives has spread right round the world. Yet even this grim necessity has brought with it some measure of compensation, in drawing closer the bonds of friendship, between the men of science in the countries thus united in a common purpose. We in Britain received a tremendous encouragement in the early months of this year, from the visit of President Conant and his associates to establish here, in London, an office for the maintenance of regular and intimate co-operation, between the war researches of our American colleagues and those which are here in hand. More recently, and in spite of all difficulties of communication, the sense of a common peril and a common determination is bringing us into a new and growing intimacy of collaboration with our colleagues of Soviet Russia. The organization of the science of the British Empire for war has brought to London already a number of distinguished colleagues from the Overseas Dominions, and we have heard of others who are on the way. It has been a particular pleasure to gather them here, in the house of the Royal Society, and to invite them to regard it as a centre and a rallying point for discussion of the means by which this new and closer collaboration, arising under the stimulus and the necessity of war, may be perpetuated and strengthened for the purposes of peace.

Generous gifts to the Society, during the year, from sister Societies in America, have given further welcome evidence of the determination of our colleagues there to come to the help of British science in this time of need. The American Philosophical Society, founded in 1743 by Benjamin Franklin, with this Society as his model, sent us ten thousand dollars 'for the aid of science in Britain'. They have confirmed our interpretation of their fraternal gift, as betokening a desire to help us to preserve some measure of normal scientific activity in this country during the war, and to keep alive the tradition of a free pursuit of knowledge for the benefit of all men. We have been able to find good use already for a large part of this benefaction, in the maintenance of a number of important researches, which war conditions had threatened to interrupt or to bring to an end. The American Physiological Society similarly sent us five thousand dollars, as a spontaneous contribution, to the support of scientific publication in Britain, mentioning Physiology as the subject to which they, as physiologists, desired us to give the first consideration. The Rockefeller Foundation, that truly international benefactor and promoter of natural knowledge, had already asked us to be reponsible for the distribution of twelve thousand five hundred dollars in aid of scientific publication in Britain in these difficult times.

As I have taken occasion earlier to state, gifts such as these, welcome for their own intrinsic value and for the practical uses which we are readily finding for them, are even more welcome on account of the evidence that they bring, of the feeling of comradeship between our American colleagues and ourselves. We can do no less, I think, than assure them of our determination that this closer sense of unity in aims and ideals, with them as with our fellow citizens of the British Overseas Dominions, shall not be lost, but rather strengthened, when we face together the new problems which the end of the war will bring.

Though the first and imperative call on the science of all free countries is for the means of winning the war, to save the freedom without which science cannot in any true sense survive, we cannot put aside the duty of preparing for the part which science must play, in rebuilding and maintaining civilization when peace returns. The Conference recently organized by the British Association, on 'Science and World Order', attracted more attention from the Press and the public than is usually given to scientific events and discussions; and it was, indeed, an impressive and significant fact that men of science from a dozen or more different countries, some far distant, should have found it possible now to meet, here in our war-scarred London, and to find the time and the impulse for such debate. We may offer our very sincere congratulations on the success of such an enterprise. Many who took part in these meetings, held at a time when Science finds itself conscript and organized as never before for the destructive purposes of war, were clearly ready to support the view that it should be as fully organized by the governments of a world at peace, for its proper purposes of enriching life and enlarging the opportunities of happiness for all men alike. There were not wanting voices, however, such as that of our Biological Secretary, to sound a warning of dangers which might be entailed, by such fullness of association between science and government as others were advocating with conviction and enthusiasm. Freedom and opportunity, it was pointed out, rather than organization, provide the conditions for the highest types of research, and thus, in the end, for the greatest services which science can give to mankind. I find myself in sympathy with this view, and nobody here, I think, would suggest that it is usually possible to organize the researches which advance boldly into the unknown, and open new vistas to human understanding. Here we shall certainly not overlook the fact that, in the period between the two wars, important funds have been placed at the disposal of the Royal Society by a series of generous benefactors, to be administered for the support of researches over a wide range of subjects, in complete independence of any control by the State.

On the other hand, I think that it will be agreed that the remarkable development in this country, since 1914, of the State support of research administered by the three Research Councils, normally in relation to the needs and the activities of a nation at peace, has taken place without any obvious detriment or danger to the freedom of science. The Royal Society's former function, of advising the Government directly on all scientific matters, and of organizing such systematic researches as were then undertaken in the public interest, has, of necessity, been shared and greatly diminished. We, as a Society, however, can fairly regard this development as, in many respects, a realization of the plans and the dreams of our predecessors here; and I do not think it fanciful to claim that our Society's traditions and standards have been still effective, through the influence of our Fellows on the Research Councils and their Committees, and through the filling of their chief executive offices by men of our Fellowship. As a whole-time research worker myself, since 1914, under the body which became the Medical Research Council, and the senior now in that service, I can bear grateful witness to the freedom of opportunity which can exist under an enlightened organization and control, exercised on behalf of the Government. I have no reason to suppose that the conditions are otherwise under the other Research Councils. Nor should we lose sight of the fact that a further large proportion of the free scientific research of the country is now indebted to support from the State through grants to the Universities, administered without any trace of detailed Government control.

While, therefore, the existing mechanisms for the support of science by the State are doubtless susceptible of improvement at one point or another, I find no reason to fear any threat to the freedom of science from them, or from any natural development on those lines. Nor do I fear it from a wider use of the organized application of science and scientific method to problems of public welfare; nor, again, from a more effective access of scientific knowledge to those responsible for government. A year ago Sir William Bragg told the Society of the formation of the Scientific Advisory Committee to the War Cabinet, under the chairmanship of Lord Hankey, with the President and two Secretaries of this Society as members *ex officio*. The representation of the Society has, indeed, been strengthened since then, and in a manner most welcome, by the fact that, though I have succeeded him *ex officio*, Sir William Bragg still gives his wisdom and experience to the work of that Committee, as an extra member.

There is one direction, however, in which I do find some reason to fear for the freedom of science. If science should become entangled in controversial politics, through the over-eagerness of its advocates and champions to invoke the sanction of science, or to claim its potentialities, in support of any special political doctrine, then indeed I believe that the threat to its freedom might become a real danger. Let there be no misunderstanding of my meaning. I am not abusing the privilege of this Chair by using 'controversial' as an epithet, to be applied to political opinions which I do not happen to share. I see danger if the name of science, or the very cause of its freedom, should become involved as a battle cry in a campaign on behalf of any political system, whether its opponents would describe it as revolutionary or reactionary. If science were allowed thus to be used as a weapon of political pressure, it would be impossible to protect science itself eventually from the pressure of sectional politics. If that should happen the dangers are, I believe, beyond dispute—the danger, for example, that fundamental researches, having no immediately practical appeal, would be allowed to fall into arrears through relative neglect; or the danger that the rigid standards of true science would be relaxed, by allowing the convenience of results for policy or for propaganda to enter into the assessment of their validity as evidence. This Society, with its firm and unbroken tradition of complete aloofness from political controversy, may still find it an important part of its function, to keep watch and, if necessary, to stand without compromise, for the right and the duty of science to seek the truth for its own sake, in complete freedom from any kind of extraneous influence. I hope, indeed, that there will never be need thus to invoke our tradition, in order to protect the freedom and the integrity of science from the enthusiasm and the advocacy of any of its friends.

Awards of Medals, 1941

The COPLEY MEDAL is awarded to Sir THOMAS LEWIS.

Lewis's life's work, still in vigorous progress but for interruption by war duties and war conditions, has been the application of precise and controlled methods of experimental research to problems of Clinical Medicine. This has enabled him to achieve a detailed analysis of abnormalities of function produced by disease, injury, or hereditary defect; and so far his attention has been centred upon the circulation of the blood and its disorders. Being attracted through the work of the late Sir James Mackenzie to the study of abnormal rhythms of the human heart-beat, Lewis recognized, about 1908, the great opportunity for a closer investigation of them offered by the then recent introduction of the string galvanometer by Einthoven. With its aid Lewis had soon made a number of clinical and laboratory studies, such as those in which he finally identified auricular fibrillation as the cause of a particular kind of complete irregularity. He was thus led to undertake, and to extend, with a succession of collaborators from many countries, the remarkable series of investigations, carried through in logical sequence between 1910 and 1923, in which he passed from the laboratory to the clinic and back again as the occasion demanded. It is fitting that special mention should here be made of the series of experimental studies published in our own *Philosophical Transactions* from 1914 to 1916, and presented in brilliant summary by Lewis in his Croonian Lecture to the Society in 1917. In these were traced, with an astonishing precision of measurement and timing, the point of origin and exact course of the rhythmical waves of excitation and contraction in the normally beating heart of the dog, and, finally, for comparison, in the hearts of other classes of vertebrate animals. Considered by itself, this work ranks as one of the outstanding achievements of experimental physiology in our times, and it has given to physiology a large part of its present detailed knowledge of the nature of the heart-beat. For Lewis, however, its greater importance lay in giving to clinical medicine the background for an accurate picture of disturbances of the normal mechanism, therewith a new security of diagnosis and prognosis in dealing with disordered actions of the heart, and ultimately a rational basis for their treatment. A new phase of cardiological thought and practice spread rapidly from Lewis's clinic round the world.

Meanwhile he had begun in 1917, and was to maintain with a series of collaborators for more than another decade, a separate series of investigations, dealing by direct experiment with the blood vessels of the human skin. Thus were elucidated the means by which the resistance of these vessels to the flow of blood is maintained and varied, including their complex reactions to chemical substances akin to histamine, which he proved to be released from the cells of the epidermis by injurious or irritant stimuli. These methods of investigation were later developed and extended to vascular disorders of the limbs, and the experiments of still more recent series have dealt with pain and functional defects in muscles and nerves, due to interruption of the blood supply. Apart from the separate accounts of items and stages of these lines of research, as completed, in papers which have issued from his department in steady sequence, Lewis has assembled and discussed the results, in their appropriate connexions, in a succession of comprehensive monographs. He has been the inspiring leader of a group of younger workers in Clinical Research as an experimental science, has founded a Society for such studies and has devoted to their use a Journal which he had started with a more limited scope.

The work of Thomas Lewis, which we honour to-day with the highest recognition in the gift of the Royal Society, is renewing and carrying forward, with a special directness, the great tradition which William Harvey created, before this Society was founded.

A ROYAL MEDAL has been awarded to Professor EDWARD ARTHUR MILNE.

Milne is distinguished for his work on planetary and stellar atmospheres, on the internal constitution of the stars, and on the theory of relativity. An early paper on various properties of the earth's atmosphere, up to high levels, led later to a valuable improvement of the theory of the escape of planetary atmospheres by the passage of the faster moving molecules out of the range of the gravitational field. Thence he passed on to a long series of investigations on radiative equilibrium and the theory of the atmospheres of the sun and stars.

Next, partly in collaboration with R. H. Fowler, he improved and extended Saha's theory of the absorption lines in stellar spectra, obtaining a relation between the maximum strength of a line and the mean pressure and temperature in the atmosphere. Afterwards Milne generalized this theory, providing a rational foundation for the astronomers' empirical method of determining stellar parallaxes from the spectra.

Later he considered the equilibrium of the calcium chromosphere of the sun, and also discovered a method by which outward moving atoms capable of absorbing radiation in the region of the chief absorption lines can be accelerated and pass beyond the range of the sun's attraction, attaining a limiting speed of about 1000 miles a second. This theory also has an application to 'new' stars.

Milne then considered the deeper layers of the atmosphere (*e.g.* in the case of the sun, the photosphere), which in 1929 he took as the subject of his valuable Bakerian lecture. He has also given a theory of the structure of sunspots and of the circulation associated with them.

In 1929 he began a new series of researches on the radiative equilibrium of gas spheres, designed to improve the theory of the internal constitution of the stars. His work in this field was specially important in focusing attention on the properties of degenerate matter.

In 1932 Milne began an altogether different series of investigations, bearing on the largest topics of astronomy and cosmogony, and providing an alternative to the general theory of relativity developed by Einstein and his followers. He has made a valuable analysis of the concept of time, and his kinematical theory of gravitation has some promise of a possible extension to include also electromagnetism.

Milne's later work has been the subject of much controversy: but the originality and boldness of his attack seem certain to promote our understanding of these great problems, and to open up fresh views and methods of approach to them.

A ROYAL MEDAL is awarded to Professor ERNEST LAURENCE KENNAWAY.

Kennaway is the director of the Chester Beatty Research Institute of the Royal Cancer Hospital, and has been engaged for the past twenty years in investigations on the production of cancers by the continued effects of chemical agents. The long known liability to skin cancer of men whose work involves regular contact with soot, coal tar, or pitch, and the more recently demonstrated production of such cancers in animals by painting the skin with tar, had raised the question whether a specific, chemical agent was concerned, or only a sufficiently persistent irritation. The fractionation of coal tar, in search of a substance revealing its action only after tedious months of experiment, was an undertaking to daunt any but a courageous and devoted investigator. Kennaway embarked on this unpromising quest, and after years of patient and tenacious labour the fluorescence studies of his co-workers Mayneord and Hieger made it possible to isolate a pure substance from tar with intense carcinogenic activity, and ultimately to identify it as benzpyrene. This identification and its confirmation by synthesis were due to J. W. Cook. Meanwhile, the observation that active fractions of the tar had ultraviolet fluorescence spectra resembling those of hydrocarbons of the phenanthrene type led to the testing of various compounds of this nature. The first of these was a dibenzanthracene, which had been synthesized by Clar, and this was found to be carcinogenic. Substances of still greater activity have since been obtained, and some of these have a close structural relationship to naturally occurring sterols. A characteristic nuclear ring-complex thus, surprisingly, provides a link of structural community between these carcinogenic agents and a number of natural substances having intense but widely different biological activities—the D vitamin, a number of hormones concerned with sexual functions, and the highly toxic but remedially important glucosides of the digitalis series. One of the most fascinating chapters in organic and biological chemistry has been opened. Identification of these provocative agents does not tell us how they act, and the causes of seemingly spontaneous cancers remain as yet obscure. It cannot be doubted, however, that, when further advances of knowledge enable a correct account to be written of the nature and mode of origin of the malignant tumours, the pioneer work of Kennaway and his able group of co-workers will provide one of the important chapters.

The DAVY MEDAL is awarded to Dr HENRY DRYSDALE DAKIN.

Dakin began his researches in the field of biochemistry early in the present century. At that time, in comparison with knowledge of the end products of metabolism, relatively little was known about the chemical activities of the living cells of the animal body. His work has made very important additions to knowledge of the intermediary changes produced by these activities, and also of the chemical structure of natural components of the tissues.

One side of Dakin's work has dealt with enzymes of the animal organs. He was the first to show that such an enzyme will attack at different rates the two optical isomers in a racemic mixture. With Kossel he discovered the enzyme arginase, with its important role in the production of urea from arginine. Later he discovered the enzyme glyoxalase, the wide distribution of which in the tissues must indicate some important though still undefined function in carbohydrate metabolism.

In connexion with the intermediary metabolism of fatty acids, Dakin produced the first convincing evidence for oxidation at the β-carbon-atom as the first stage of their utilization by the body, and showed that this type of oxidation can even be reproduced *in vitro* by the action of hydrogen peroxide.

Dakin's work on the chemistry of the proteins has included a method of partial racemization, bringing subtle differences of molecular pattern into view, which could be related to specific antigenic differences. He also introduced a method of separation which enabled new hydroxyaminoacids to be recognized, and raised much nearer to unity the proportion of a protein-molecule accounted for as known amino-acids. At a wide interval of years, Dakin has made two notable contributions to the chemistry of hormones. In 1905, he was responsible for the first published artificial synthesis of a hormone, adrenaline. In 1936 he described the isolation from liver of a substance which is, at least, a principal factor in the important effect of liver extracts on pernicious anaemia.

Working in France during the war of 1914–18, Dakin introduced the use of a buffered hypochlorite solution for irrigating infected wounds. Later, on a ship bringing the sick and wounded from Gallipoli, he made a similar solution by direct electrolysis of sea-water. Another war is reviving the use of such preparations to meet the same and other needs.

Though Dakin has worked in a private laboratory, with but rare collaboration and no pupils, his work has exercised a wide and powerful influence on the growth of biochemistry and on the strengthening of its links with organic chemistry.

The HUGHES MEDAL is awarded to Professor NEVILL FRANCIS MOTT.

Early in his career Mott's work in the field of atomic collisions attracted widespread attention. He was the first to show that Rutherford's scattering law holds in quantum mechanics, and to give an accurate theory of the effect of symmetry in scattering problems. When he took up his present post as Professor of Theoretical Physics at Bristol, he abandoned this subject for that of the theory of metals and alloys. Within a few years he was recognized as one of the leading international authorities in this field, to which he contributed a number of important papers on electronic bands in metals and on the electrical conductivity of alloys and its temperature coefficient. In establishing a close association between theoretical and experimental workers he has no superior. He has also contributed to the important problem of metals under strain; under his direction Fuchs has calculated the elastic constants of certain metals and obtained good agreement with known values.

Later he turned to semi-conductors and insulators, throwing light on the physical processes involved in the formation of oxide films, and in the electrical conductivity which can be induced by various means in polar crystals. His theory (with Gurney) of the formation of the latent image in a photographic emulsion has found general acceptance, and has stimulated fresh experimental work in research departments of the industry.

This striking record of published work was achieved between 1928 and 1939.

When war broke out Mott found that his activities as a quantum physicist had no direct application to the war. It is a tribute to his versatility and flexibility of mind that he could turn so quickly to a new field in classical physics, and make important contributions to the practical problem in connexion with which he now holds an important post.

Clarke Memorial Medal (1941)—*Royal Society of New South Wales*

The Royal Society of New South Wales has awarded the Clarke Memorial Medal for 1941 to FREDERIC WOOD-JONES, F.R.S., now Professor of Anatomy in the University of Manchester. Professor Wood-Jones being unable to attend at Sydney, we have been asked, by our daughter Society of New South Wales, to arrange for the medal to be presented to him here on an appropriate occasion. We have judged that none could be more appropriate than this, on which our own medals are presented, and it gives me just pleasure to be able to make the presentation. The Royal Society of New South Wales have stated the grounds of the award in the following terms: 'The Clarke Memorial Medal, established as a memorial to the late Rev. W. B. Clarke, who for nearly forty years rendered distinguished service to his adopted country, Australia, is awarded "for distinguished work in the Natural Sciences done in or on the Australian Commonwealth and its territories." Professor Wood-Jones is receiving the distinction on account of his very outstanding researches in zoology in Australia, particularly for his work on the mammals of South Australia. Moreover, during his stay in Australia Professor Wood-Jones not only did much research work in South Australia and Victoria, but also took a leading part in scientific endeavour in general.'

The inhibition of growth by chemical compounds

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In an extended investigation of the growth-inhibitory activity of carcinogenic compounds and related substances, over two hundred compounds were tested, including various 5-, 10- and 9:10-substituted benzanthracenes, dimethyl derivatives of anthracene, nitrogenous analogues of 1:2-benzanthracene, benzphenothiazines and dibenzphenothiazines, compounds related to 3:4-benzphenanthrene, dibenzfluorenes, dibenzcarbazoles, dibenzpyrenes, azonaphthalenes and related products, naphthylamines and naphthaquinones, arsenonaphthalenes, derivatives of triphenylethylene, and diphenyl derivatives of indene, *β*-naphthindole and *β*-naphthofuran.

A striking degree of correspondence was often shown by the inhibitory and carcinogenic activity of closely related compounds (e.g. 5-alkyl benzanthracenes; dibenzfluorenes; dibenzphenanthrenes; 2:2′-azonaphthalene, 2:2′-diamino-1:1′-dinaphthyl and 3:4:5:6-dibenzcarbazole). However, no inhibitory activity was observed for certain carcinogenic 10- and 9:10-substituted benzanthracenes. On the other hand, inhibitory activity was noted in a few compounds (e.g. 1:2′-azonaphthalene) which have yielded few or no tumours in exhaustive tests, and in some of a group of synthetic oestrogenic compounds which, although not carcinogenic in the usual sense, are nevertheless associated with the induction of individual types of tumour under special conditions.

The relation between molecular structure and inhibitory activity depends in general upon an optimal degree of molecular complexity and upon certain more specific requirements. Nevertheless, the results obtained with derivatives of triphenylethylene suggest that inhibitory activity may still be shown by compounds diverging widely from the polycyclic structure and possessing only a skeletal resemblance.

Diminution of inhibitory effect with increased substituent size was shown in the 5-alkyl benzanthracenes tested, although the same relation does not necessarily obtain for other positions. The influence of the nature of the substituent is seen (for example) in the contrast between 10-methyl-, 10-amino- and 10-cyano-1:2-benzanthracene (inhibitory) and 10-*iso*propyl-1:2-benzanthracene (inactive). Lastly, numerous experiments indicated that solubilization of an active compound usually entails decrease of activity, although certain apparent exceptions were encountered.

In addition to the relationship between inhibitory activity and carcinogenicity, and that between both biological properties and chemical structure, consideration is also given to the mode of production of the inhibitory effect.

1. Introduction

In an investigation of the action of tumour-producing compounds it was found that many carcinogenic hydrocarbons retard the growth of tissues both normal and malignant. Extended investigation showed a statistical association, of high significance, between the biological properties of carcinogenicity and growth-inhibitory power (Haddow 1935, 1938*a*; Haddow and Robinson 1937, 1939; Haddow, Scott and Scott 1937). It was further suggested that the association is of etiological importance, and tumour induction by these compounds was conceived to take place in two stages: (1) interference with the growth of normal cells and (2) an adaptive cellular reaction—accomplished mainly by dedifferentiation—in virtue of which the altered cells are able to achieve independent multiplication, at an increased rate, in an environment which makes normal growth difficult or impossible for their parent cell. While the new growth characters appear in response to a specific pathological change in the cellular *milieu*, the cellular changes themselves are permanent: hence they continue to be manifested, indefinitely and without reversion, when the variant cells reach normal tissues either in the same host or artificially in other hosts by transplantation. The direct and collateral evidence supporting this interpretation has been fully reviewed elsewhere (Haddow 1937, 1938 *b*, *c*). The principle does not appear to be limited to the mechanism of carcinogenesis by chemical compounds, but is probably of wider application; in particular, it was suggested as an explanation of the apparent paradox that X-rays and gamma-rays, as is the case with the carcinogenic hydrocarbons, exhibit both tumour-producing and growth-inhibitory activity. In addition to papers confirming the original observation, and alluded to in the references already given, three contributions of more recent date may be mentioned, viz. by Morelli and Dansi (1939), Dansi, Morelli and Sempronj (1940), and Davis, Murphy and Emge (1941).

At an early stage it was recognized that carcinogenicity depends on optimal rather than maximal inhibition. This fact, and a theoretical consideration to be dealt with in the discussion, held out the prospect of discovering compounds possessing enhanced inhibitory activity but with carcinogenicity restricted, minimal or even extinguished. The present paper records an extension of this investigation; while it has revealed many new points of interest in relation to the mode of action of carcinogenic agents, progressively more attention has been paid to its inherent possibilities as a rational approach to the chemotherapy of cancer.

2. EXPERIMENTAL METHODS

Assay of the growth-inhibiting activity of individual compounds was made by means of a standard experiment. Two groups each of twelve to fifteen albino rats from a colony maintained for the purpose, carefully selected as to individual weight (*c.* 100–120 g.), were implanted with the Walker carcinoma 256. The technique of implantation was similar to that previously described. On the following day, animals of the trial series received a single dose, in many cases determined by a preliminary toxicity experiment, of the compound for test (in sterile solution or suspension in arachis oil or in water), by intraperitoneal injection; the control series received an equal volume of the solvent alone. Since the primary object of these experiments was the detection of relatively high activity, and not the determination of absolute presence or absence of inhibitory power, doses employed were as a rule only 50 % of those employed in earlier work (Haddow and Robinson 1937).* An estimate of activity was obtained by comparing the mean weights of the two series of tumours, after their removal at the end of a suitable interval (in most cases 14 or 15 days). It is believed that the experimental error in the primary data is very small, and certainly as small as can be attained. The significance of any difference between the means was calculated by the t test and also, in certain cases where two or three trial series possessed common controls, by an analysis of variance.

It is a common impression that transplanted tumours are less reliable test objects than are spontaneous tumours, in experiments of this type. While this is probably true for many transplantable tumours, it does not necessarily apply in the case of such tumours as the Walker rat carcinoma 256 or the Crocker mouse sarcoma 180. The former has proved comparatively resistant to cure by X-radiation, and the latter is remarkably refractory to inhibition of any kind. On the other hand, spontaneous adenocarcinomata of the mamma in the mouse were found in individual cases to be surprisingly sensitive to chemical inhibition (Haddow 1938*a*), and, although the range of sensitivity is wide, many instances of regression were observed. Thus, while every prospective chemotherapeutic substance must be tested by its capacity to inhibit spontaneous neoplasms, it need not be assumed that these tumours are more refractory than the transplanted tumours mentioned. The use of a reliable propagated tumour also enables closely controlled experiments to be carried out as extensively as may be wished, and thus facilitates the examination of large numbers of compounds on a sufficiently accurate basis.

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* *Note, added* 19 *August* 1941. Such dosage lies within the range commonly employed in tests for carcinogenic activity by subcutaneous injection, but is considerably greater than the least quantity of a potent compound sufficient to produce a high yield of tumours (see for example Shear and Leiter 1941).

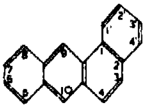
3. RESULTS

(a) *Derivatives of* 1:2-*benzanthracene*

(i) 5-*alkyl derivatives*.

It has been recognized for some considerable time that alkyl substitution in position 5 of the benzanthracene molecule is an important determiner of both carcinogenic and growth-inhibitory properties (Barry, Cook, Haslewood, Hewett, Hieger and Kennaway 1935; Haddow and Robinson 1937).

TABLE 1. 5-ALKYL BENZANTHRACENES

experiment number	type  1' 2' 3' 4' 1 2 3 4 5 6 8 9 10	dose mg.	mean tumour weights (g.) control series	mean tumour weights (g.) treated series	ratio of means	*t*	*P*
1	5-methyl-1:2-benzanthracene	25	24·54	5·56	4·41	4·85	0·001
2	5-ethyl-1:2-benzanthracene	25	24·54	7·29	3·37	4·56	0·001
3	5-*n*-propyl-1:2-benzanthracene	25	24·54	7·55	3·25	4·09	0·001
4	5-*n*-butyl-1:2-benzanthracene	25	24·54	16·81	1·46	1·72	0·1
5	5-*n*-amyl-1:2-benzanthracene	25	28·02	23·35	1·20	0·95	0·4
6	5-*n*-hexyl-1:2-benzanthracene	25	28·02	16·94	1·65	2·57	0·02
7	5-*n*-heptyl-1:2-benzanthracene	25	28·02	21·40	1·31	1·21	0·3

It seemed of interest to ascertain the effect of extension in length of the substituent, and a complete examination was made of seven 5-alkyl derivatives comprising the homologous series 5-methyl-, -ethyl-, -*n*-propyl-, -*n*-butyl-, -*n*-amyl-, -*n*-hexyl- and -*n*-heptyl-1:2-benzanthracene (see Cook and Robinson 1940). The records given in table 1* show maximal inhibitory activity in the first member of this series, and the trend of the results indicates very clearly a progressive decline in activity as the number of carbon atoms in the chain is increased to four; subsequent additions produced no further diminution which could be measured. Data have been accumulated (see Badger, Cook, Hewett, Kennaway, Kennaway, Martin and Robinson 1940) regarding the carcinogenic effect of these compounds on the skin of mice, and similar although less complete information is available in respect of their sarcoma-producing capacity. These data are sufficiently extensive to enable an index of carcinogenicity to be estimated for most of the compounds according to the method of Iball (1939), and figure 1 affords a comparison of the changes produced by increasing the substituent chain length (1) on growth-inhibitory activity and (2) on the power to induce epithelioma in mice.

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* As the original data on which tables 1–20 are based are too extensive for publication, a copy has been deposited with the Librarian, General Library, British Museum (Natural History), London, S.W. 7.

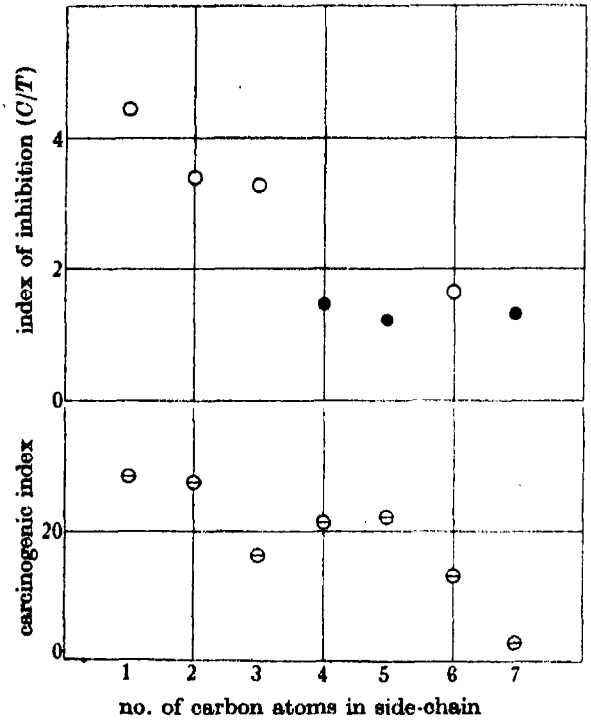
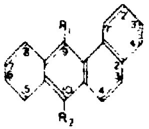
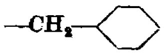
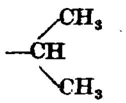
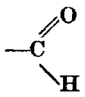


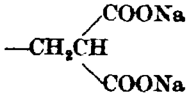
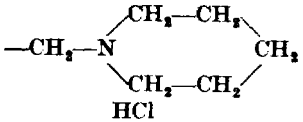
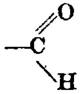
Figure 1. Inhibitory and carcinogenic activity of 5-methyl-, 5-ethyl-, 5-*n*-propyl-, 5-*n*-butyl-, 5-*n*-amyl-, 5-*n*-hexyl- and 5-*n*-heptyl-1:2-benzanthracene.

○ inhibition significant; ● inhibition not significant.

Both the index of inhibitory activity and the carcinogenic index show a significant downward trend as the number of carbon atoms in the side-chain

TABLE 2. 10- AND 9:10-SUBSTITUTED BENZANTHRACENES

experiment number	type			dose mg.	mean tumour weights (g.)		ratio of means	*t*	*P*
	R_1	R_2			control series	treated series			
8			10-benzyl-1:2-benzanthracene	13·5	20·23	18·64	1·09	0·63	0·6
9		 $—CH(CH_3)_2$	10-*iso*propyl-1:2-benzanthracene	25	20·23	20·02	1·01	0·09	1·0
10		 $—CHO$	1:2-benzanthracene-10-aldehyde	25	20·23	8·93	2·27	4·47	0·001
11		$—CO.CH_3$	10-acetyl-1:2-benzanthracene	25	15·33	19·85	0·77	−1·10	0·3
12		$—CH_2.O.COCH_3$	10-acetoxymethyl-1:2-benzanthracene	13·5	26·99	24·38	1·11	0·94	0·4
13		$—CH_2CH_2OH$	10-*β*-hydroxyethyl-1:2-benzanthracene	25	9·30	7·29	1·28	0·55	0·6
14		$—NH_2$	10-amino-1:2-benzanthracene	25	22·24	7·74	2·87	5·11	0·001
15		$—CN$	10-cyano-1:2-benzanthracene	25	21·96	10·87	2·02	2·33	0·05
16		$—CH_2COONa$	sodium 1:2-benzanthranyl-10-acetate	10	24·52	23·79	1·03	0·19	0·9

17		$—CH_2CH_2COONa$	sodium-β-(1:2-benz-10-anthranyl)-propionate	25	**12·43**	**10·28**	1·21	0·53	0·6
18		 $—CH_2CH(COONa)_2$	sodium-(1:2-benzanthranyl-10-methyl)-malonate	25	**12·43**	**6·80**	1·83	1·41	0·2
19		$—CO.COONa$	sodium 1:2-benzanthranyl-10-glyoxylate	25	**24·52**	**23·37**	1·05	0·30	0·8
20			1:2-benzanthranyl-10-methylpiperidinium hydrochloride	25	**12·43**	**14·08**	0·88	−0·42	0·7
21	$—C_2H_5$	$—C_2H_5$	9:10-diethyl-1:2-benzanthracene	25	**19·88**	**24·49**	0·81	−1·01	0·4
22	$—CH_2OH$	$—CH_2OH$	9:10-bishydroxymethyl-1:2-benzanthracene	8	**19·58**	**20·14**	0·97	−0·14	0·9
23	$—CH_2OCO(CH_2)_2CO_2Na$	$—CH_2OCO(CH_2)_2CO_2Na$	disodium-disuccinate of 9:10-bishydroxy-methyl-1:2-benzanthracene	25	**24·52**	**23·23**	1·06	0·39	0·7
24	$—CH_2OCOCH_3$	$—CH_2OCOCH_3$	9:10-bisacetoxymethyl-1:2-benzanthracene	14·5	**19·58**	**30·73**	0·64	−2·30	0·05
25	$—CH_3$	 $—CHO$	9-methyl-1:2-benz-10-anthraldehyde	10	**9·30**	**5·54**	1·68	1·10	0·03
26	$—CH_3$	$—CN$	9-methyl-10-cyano-1:2-benzanthracene	10	**22·24**	**25·44**	0·87	−0·91	0·4

increases. The correlation coefficient between the two measures is 0·59, but, owing to the small number of observations, is not significant. The data respecting inhibition are derived from two experiments, the compounds being tested in two groups, of four and three. It is known that both experiments were of good quality, and it seemed justifiable to plot the mean tumour weights as these correspond to the seven substituents. The upward trend of these mean weights is significant, and there is an average increase in tumour weight of 2·95 g. for each carbon atom in the chain. While the significant upward trend of the mean weights is all that can be established, the plotted means suggested that the real relationship is akin to a growth curve, and a logistic curve yields a moderate fit.

(Mean tumour weight $(W) = \frac{24{\cdot}5}{1 + 6{\cdot}39e^{-0{\cdot}596\,C}}$, where C denotes the number of carbon atoms in the side-chain.)

This curve gives a significantly better fit than the best horizontal line, and a better fit (although not significantly) than a slanted line.

(ii) 10- *and* 9:10-*substituted benzanthracenes.*

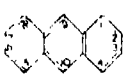
Inhibitory activity has already been reported for the carcinogenic hydrocarbons 9-methyl-, 10-methyl- and 9:10-dimethyl-1:2-benzanthracene (Haddow and Robinson 1939), and tests have now been completed for a series of related compounds certain of which possess functional groups conferring, in some cases, solubility in water (Badger and Cook 1939, 1940). The details of these experiments are presented in table 2. It is apparent that the introduction of hydroxyl and carboxyl groups is attended by marked loss of growth-inhibitory activity, and in no case was any augmentation observed. Significant inhibition was shown by the carcinogenic compounds 10-amino- and 10-cyano-1:2-benzanthracene and 1:2-benzanthracene-10-aldehyde (Shear and Leiter 1940), and was absent in the case of the non-carcinogenic 10-benzyl-, 10-*iso*propyl- and 9- or 10-acetyl- derivatives. On the other hand, four carcinogenic compounds (10-β-hydroxyethyl-, 10-acetoxymethyl-, 9 : 10-bishydroxymethyl- and 9-methyl-10-cyano-1 : 2-benzanthracene) gave no trace of inhibition. While this is possibly due to the unusually low dosage employed in the individual experiments, certain of these cases constitute real anomalies, and it should be recalled that no inhibitory activity was discovered in the case of 10-hydroxymethyl-1:2-benzanthracene (Haddow and Robinson 1939). It may be noted that 1:2-benzanthracene-10-aldehyde was found by Dansi and Vercellone (1939) to be reduced by fermenting yeast to the 10-hydroxymethyl compound. The case of 9:10-bisacetoxymethyl-1:2-

benzanthracene is of special interest since the dose employed produced a significant stimulation of growth. The mechanism by which such an effect may be caused has been discussed previously (Haddow, Scott and Scott 1937, pp. 498–9).

(*b*) *Dimethyl derivatives of anthracene*

Since methyl substitution at favourable positions in 1:2-benzanthracene greatly enhances both inhibitory power and carcinogenicity, experiments were carried out with one tetramethyl and five dimethyl derivatives of anthracene, which had itself been found devoid of growth-inhibitory or carcinogenic activity (table 3). Some inhibitory activity was detected with 1:3-dimethylanthracene, although no tumours have been obtained.

TABLE 3. DIMETHYL DERIVATIVES OF ANTHRACENE

experiment number	type 	dose mg.	mean tumour weights (g.) control series	mean tumour weights (g.) treated series	ratio of means	*t*	*P*
27	1:2-dimethylanthracene	25	**25·58**	**24·05**	1·06	0·51	0·7
28	1:3-dimethylanthracene	25	**28·02**	**17·05**	**1·64**	2·08	0·05
29	1:4-dimethylanthracene	25	**19·88**	**19·37**	1·03	0·11	1·0
30	2:3-dimethylanthracene	25	**23·86**	**22·75**	1·05	0·22	0·9
31	9:10-dimethylanthracene	25	**7·34**	**11·59**	0·63	– 1·03	0·4
32	9:10-dimethylanthracene	25	**6·40**	**11·89**	0·54	1·69	0·2
33	2:3:6:7-tetramethyl-anthracene	25	**19·88**	**15·65**	1·27	0·90	0·4

Also, while carcinogenic activity had been shown by 9:10-dimethylanthracene (slow development of three epitheliomata) no inhibition was observed. It is obvious that 1:2:9:10-tetramethylanthracene would be of greater interest, on account of its closer relation to a benzanthracene substituted in positions conferring pronounced carcinogenic action. So far this compound remains unknown; three unsuccessful attempts to synthesize it have already been reported (Badger, Cook and Goulden 1940; Fieser and Webber 1940; Badger, Goulden and Warren 1941). Compounds previously tested for carcinogenic activity, with negative results, include 1-phenyl, 2-phenyl, 9:10-dibenzyl and 1:5-di*iso*propenylanthracene (Cook 1932).

(*c*) *Nitrogenous analogues of* 1:2-*benzanthracene, and water-soluble derivatives*

It had already been found that 1:2:5:6-dibenzacridine possesses inhibitory properties (Haddow 1938*a*), and a fuller examination was made to test the influence of methyl substitution in the inactive compound 1:2-benzacridine, at positions corresponding to 7-, 9- and 5:7-substitution in 1:2-benzanthracene (table 4). It is apparent that such substitution again confers activity, although not to a greater degree than in 1:2-benzanthracene, and possibly less. Little or no activity was demonstrable in water-soluble methosulphates of active compounds, under similar conditions of test.

'Alizarin Blue' and 'Alizarin Blue AS' represent compounds containing nitrogen in the angular ring: it may be recalled that Sempronj and Morelli (1939) found tumour nodules (? adenocarcinoma) in the kidney in two rats which had been injected with the parent compound β-anthraquinoline.

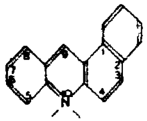
(*d*) *Benzphenothiazines and dibenzphenothiazines*

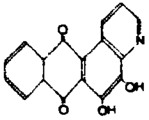
A further test of the influence upon activity (1) of changes in the central ring and (2) of alkyl substitution, is provided by the results for 1:2- and 3:4-benzphenothiazine, three methyl derivatives, and two dibenzphenothiazines (table 5). 1:2-Benzphenothiazine manifested slight activity and the 3:4- isomer none, while the N-methyl derivatives of both compounds proved inhibitory, that from the 1:2 form again appearing the more active. Considerable potency was attained in both 1:2:5:6- and 3:4:5:6-dibenzphenothiazine.

(*e*) *Compounds related to* 3:4-*benzphenanthrene*

3:4-Benzphenanthrene has already been described as producing epitheliomata after a prolonged latent period, and potency is greatly enhanced in the 2-methyl derivative (Barry *et al.* 1935; Bachmann, Cook, Dansi, de Worms, Haslewood, Hewett and Robinson 1937; Badger *et al.* 1940). As regards growth-inhibitory power, a number of experiments showed little or no activity in the parent hydrocarbon, and definite, although not great, activity in 2-methyl-3:4-benzphenanthrene (Haddow and Robinson 1939). A larger number of derivatives has now been tested (see Hewett 1938; Everett and Hewett 1940) in order to study the influence on inhibitory capacity of the position and size of the substituent. Also, experiments have been carried out with 1:2:3:4-dibenzphenanthrene, 1:2:5:6-dibenzphenanthrene, 1:2-dimethylchrysene and 1:2:3:4-tetramethylphenanthrene (table 6). The effect observed with 2-methyl-3:4-benzphenanthrene did not attain significance, and was considerably exceeded in the case of the 2-ethyl- and 2-*iso*propyl-compounds. It is of some interest that the early and characteristic histological changes produced by the carcinogenic hydrocarbons as described by Pullinger (1940) were observed by that author to develop only after large doses of 2-methyl-3:4-benzphenanthrene, and then comparatively slowly.

TABLE 4. NITROGENOUS ANALOGUES OF 1:2-BENZANTHRACENE

experiment number	type 	dose mg.	mean tumour weights (g.) control series	treated series	ratio of means	t	P
34 M 992	1:2-benzacridine	25	18·62	18·68	1·00	−0·02	1·0
35 M 993	1:2-benzacridine methosulphate	5	14·35	14·31	1·00	0·01	1·0
36 M 994	7-methyl-1:2-benzacridine	25	18·62	9·04	2·06	2·99	0·01
37 M 995	7-methyl-1:2-benzacridine methosulphate	5	14·35	14·40	1·00	−0·01	1·0
38 M 996	9-methyl-1:2-benzacridine	25	18·62	9·29	2·00	2·76	0·02
39 M 997	9-methyl-1:2-benzacridine methosulphate	10	14·35	10·09	1·42	0·9	0·4
40 M 998	5:7-dimethyl-1:2-benzacridine	25	20·91	10·00	2·09	2·09	0·05
41 M 999	5:7-dimethyl-1:2-benzacridine methosulphate	0·5	14·35	19·19	0·75	−0·88	0·4
42 M 937	'alizarin blue'	25	12·57	12·63	1·00	−0·02	1·0
43 M 938	'alizarin blue AS'	25	12·57	5·60	2·24	2·50	0·05



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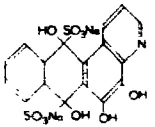

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TABLE 5. BENZPHENOTHIAZINES; DIBENZPHENOTHIAZINES

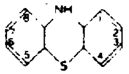
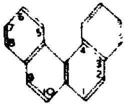
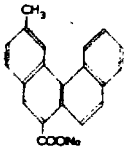
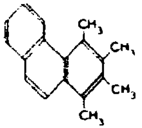
experiment number	type 	dose mg.	mean tumour weights (g.) control series	treated series	ratio of means	t	P
44 M 1500	1:2-benzphenothiazine	25	20·77	16·26	1·28	2·07	0·05
45 M 1501	3:4-benzphenothiazine	25	20·77	18·10	1·15	1·08	0·3
46 M 1502	N-methyl-1:2-benzphenothiazine	25	20·77	10·93	1·90	3·92	0·001
47 M 1503	N-methyl-3:4-benzphenothiazine	25	20·77	14·98	1·39	2·08	0·05
48 M 1504	6-methyl-3:4-benzphenothiazine	25	22·50	10·60	2·12	2·28	0·05
49 M 1504	6-methyl-3:4-benzphenothiazine	20	15·87	6·10	2·60	2·51	0·02
50 M 1505	1:2:5:6-dibenzphenothiazine	20	15·87	3·51	4·52	3·42	0·01
51 M 1247	3:4:5:6-dibenzphenothiazine	25	29·24	6·48	4·51	4·35	0·001

TABLE 6. COMPOUNDS RELATED TO 3:4-BENZPHENANTHRENE

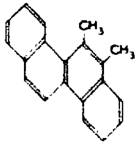
experiment number	tppe 	dose mg.	mean tumour weights (g.)		ratio of means	t	P
			control series	treated series			
52	1-*iso*propyl-3:4-benzphenanthrene	25	23·86	23·97	1·00	−0·02	1·0
53	2-methyl-3:4-benzphenanthrene	25	13·67	10·28	1·33	1·36	0·2
54	2-ethyl-3:4-benzphenanthrene	25	25·89	11·39	2·27	4·06	0·001
55	2-*n*-propyl-3:4-benzphenanthrene	25	25·89	17·25	1·50	1·66	0·2
56	2-*iso*propyl-3:4-benzphenanthrene	25	23·86	10·22	2·33	3·35	0·01
57	3:4-benzphenanthrene-2-aldehyde	25	13·58	14·55	0·93	−0·15	0·9
58	2-acetyl-3:4-benzphenanthrene	25	25·89	7·02	3·69	4·85	0·001
59	6-methyl-3:4-benzphenanthrene	25	21·96	28·00	0·78	−1·10	0·3
60	8-methyl-3:4-benzphenanthrene	25	26·99	23·72	1·14	1·09	0·3
61	sodium 6-methyl-3:4-benz-10-phenanthroate	10	26·41	20·56	1·28	1·37	0·2
62	1:2:3:4-tetramethylphenanthrene	25	15·35	15·32	1·00	0·0	1·0
63	1:2-dimethylchrysene	25	17·94	3·47	5·17	7·60	0·001
64	1:2:3:4-dibenzphenanthrene	25	15·33	4·73	3·24	2·59	0·02
65	1:2:5:6-dibenzphenanthrene	25	15·33	3·22	4·76	3·37	0·01



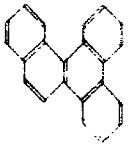
61



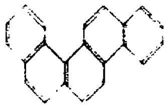
62



63



64



65

Of the tetracyclic aromatic hydrocarbons, chrysene as well as 1:2-benzanthracene and 3:4-benzphenanthrene is to be regarded as a parent compound of carcinogenic derivatives, and Hewett (1940) has summarized the development of his work on the influence of substitution in this group. These hydrocarbons are all derivatives of phenanthrene, each substituted in two of the 1-, 2-, 3- and 4-positions. Further substitution of 2:3-benzphenanthrene (i.e. 1:2-benzanthracene) in either or both of the remaining 1- and 4-positions gives rise to highly carcinogenic hydrocarbons (methylcholanthrene, 3:4-benzpyrene (figure 2, structure IV), 9:10-dimethyl-1:2-benzanthracene (structure V)), while further substitution of 3:4-benzphenanthrene in the 1- or the 2-positions (structure I), or in both (structure II), also leads to enhanced activity, as does similar substitution of the 3- and 4-positions of 1:2-benzphenanthrene giving 1:2-dimethylchrysene (structure III). In each of these cases the active compound is a phenanthrene derivative substituted in at least three of the positions 1, 2, 3 and 4. Di-substitution in these positions thus leads to little or no activity, while tri- or tetra-substitution confers marked activity. It appeared that the substitution was effective whether represented by two benzene rings (1:2:3:4-dibenzphenanthrene), or by one benzene ring and one or two methyl groups (e.g. 2-methyl-3:4-benzphenanthrene, 9:10-dimethyl-1:2-benzanthracene, 1:2-dimethylchrysene), and this consideration led Hewett to the synthesis of 1:2:3:4-tetramethylphenan-

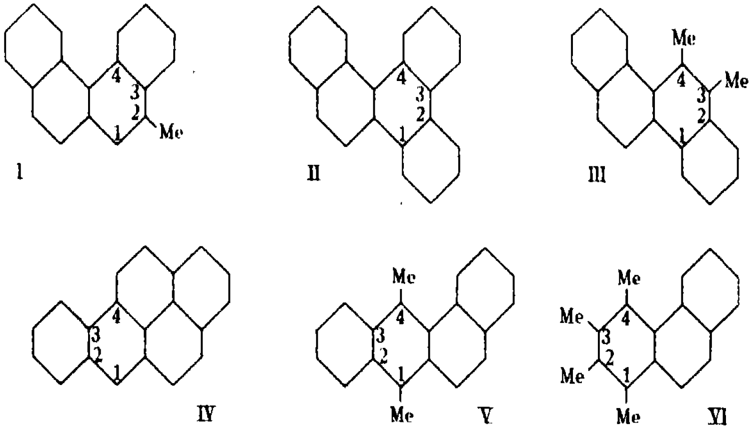


FIGURE 2. Structures derived from phenanthrene, 3:4-benzphenanthrene, chrysene and 1:2-benzanthracene (Hewett 1940). Numbering indicates relation to phenanthrene.

threne (structure VI) (Hewett and Martin 1940), since it constitutes a link between the carcinogenic derivatives of 1:2-benzanthracene, 3:4-benzphenanthrene and chrysene, and may be regarded as the prototype of all these compounds. Recent tests with this compound are almost complete, and indicate that it possesses slight carcinogenic activity: while no sarcomata appeared in twenty C3H mice each of which had received 5 mg. by injection, an epithelioma was produced in one of ten mice by application of a solution in benzene to the skin (examined at the 398th day). In the present experiments, however, 1:2:3:4-tetramethylphenanthrene showed no inhibitory effect, although pronounced activity was exhibited by 1:2:3:4-dibenzphenanthrene, 1:2:5:6-dibenzphenanthrene and 1:2-dimethylchrysene.

(*f*) *Dibenzfluorenes and related compounds*

1:2:5:6-Dibenzfluorene (carcinogenic) and 1:2:7:8-dibenzfluorene had previously been described as manifesting inhibitory activity (Haddow and Robinson 1939). At that time the latter compound was under test for carcinogenicity, and it has now been reported as active in this respect also (Badger *et al.* 1940). Table 7 presents additional results obtained for 1:2:3:4-dibenzfluorene, 3:4:5:6-dibenzfluorene (Martin 1941) and related compounds.

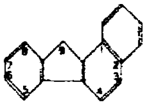
(*g*) *Dibenzcarbazoles*

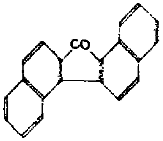
The carcinogenic action of the 3:4:5:6-, 1:2:5:6- and 1:2:7:8-dibenzcarbazoles was described by Boyland and Brues (1937). 3:4:5:6-Dibenzcarbazole was found to be growth-inhibitory (Haddow and Robinson 1939), although the effect was obscured by the marked toxicity of this compound. 1:2:5:6-Dibenzcarbazole has proved to be much less toxic and to have considerable inhibitory powers (table 8). The sodium salt of 1:2:5:6-dibenzcarbazole-N-sulphonic acid produced no detectable inhibition after a single dose but a significant effect following repeated dosage. In similar experiments the corresponding salt of 3:4:5:6-dibenzcarbazole appeared to be completely inactive.

(*h*) *Derivatives of fluoranthene*

Fluoranthene had already been reported as non-carcinogenic (Cook, Hieger, Kennaway and Mayneord 1932) and non-inhibitory, and negative results have now been obtained, in respect of inhibition, for four related compounds (table 9).

TABLE 7. DIBENZFLUORENES AND RELATED COMPOUNDS

experiment number	type  1:2-benzfluorene	dose mg.	mean tumour weights (g.) control series	treated series	ratio of means	*t*	*P*
66	1:2-benzfluorene	25	**15·33**	**15·33**	1·00	0·00	1·0
67	1:2:3:4-dibenzfluorene	25	**19·95**	**7·98**	**2·50**	3·35	0·01
68	1:2:5:6-dibenzfluorene	25	**25·58**	**6·48**	**3·95**	6·50	0·001
69 M1414	1:2:5:6-dibenzfluorenone	20	**21·60**	**2·80**	**7·71**	3·74	0·001
70	3:4:5:6-dibenzfluorene	25	**19·88**	**19·79**	1·00	0·02	1·0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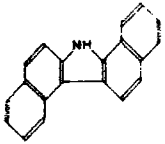

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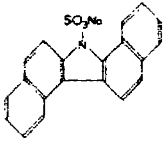

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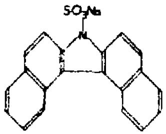

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TABLE 8. DIBENZCARBAZOLES AND WATER-SOLUBLE DERIVATIVES

experiment number	compound	dose mg.	mean tumour weights (g.) control series	treated series	ratio of means	*t*	*P*
71 M1176	1:2:5:6-dibenzcarbazole	25	**31·93**	**4·75**	**6·72**	7·85	0·001
72 M1176	1:2:5:6-dibenzcarbazole	25	**20·10**	**5·38**	**3·74**	2·69	0·02
73 M1325	sodium 1:2:5:6-dibenzcarbazole-N-sulphonate	20	**19·92**	**19·99**	1·00	−0·01	1·0
74 M1325	sodium 1:2:5:6-dibenzcarbazole-N-sulphonate	7 × 10	**21·89**	**8·60**	**2·55**	2·55	0·02
75 M1295	sodium 3:4:5:6-dibenzcarbazole-N-sulphonate	20	**19·92**	**13·30**	1·50	1·07	0·4
76 M1295	sodium 3:4:5:6-dibenzcarbazole-N-sulphonate	7 × 10	**21·89**	**20·20**	1·08	0·31	0·8

TABLE 9. DERIVATIVES OF FLUORANTHENE

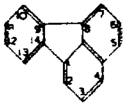
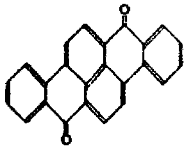
experiment number	type  fluoranthene	dose mg.	mean tumour weights (g.) control series	treated series	ratio of means	*t*	*P*
77	2:4-dimethylfluoranthene	16	21·75	18·89	1·15	0·69	0·5
78	2:3:4-trimethylfluoranthene	25	21·75	20·85	1·04	0·23	0·9
79	2:2:4-trimethyl-1:2-dihydrofluoranthene	25	21·75	21·60	1·01	0·03	1·0
80	2:2:4-trimethyl-1:2:3:4-tetrahydrofluoranthene	25	21·75	22·40	0·97	−0·15	0·9

TABLE 10. DIBENZPYRENE QUINONES

experiment number	compound	dose mg.	mean tumour weights (g.) control series	treated series	ratio of means	*t*	*P*
81 M935	3:4:8:9-dibenzpyrene-5:10-quinone	25	12·57	3·17	3·97	3·63	0·01
83 M936	leuco-3:4:8:9-dibenzpyrene quinone disulphuric acid ester (sodium salt)	25	25·65	17·73	1·45	2·00	0·05
84 M1372	3′-chloro-3:4:8:9-dibenzpyrenequinone	25	25·06	27·11	0·92	−0·63	0·6
85 M1373	3′:7′-dichloro-3:4:8:9-dibenzpyrenequinone	25	25·06	25·99	0·96	−0·28	0·8
86 M1407	leuco-3′-chloro-3:4:8:9-dibenzpyrene quinone disulphuric acid ester (sodium salt)	25	24·09	27·39	0·88	−0·68	0·6
87 M1408	leuco-3′:7′-dichloro-3:4:8:9-dibenzpyrene quinone disulphuric acid ester (sodium salt)	25	25·06	23·21	1·08	0·52	0·7
88 M972	3:4:8:9-dibenz-5:10-diazapyrene	25	11·77	6·94	1·70	2·58	0·02
90 M1073	3:4:9:10-dibenzpyrene quinone	25	29·60	15·38	1·92	2·79	0·02
91 M979	anthanthrone	25	20·71	22·86	0·91	−1·04	0·4



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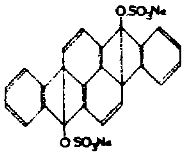

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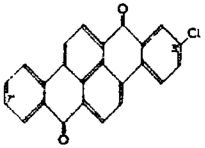

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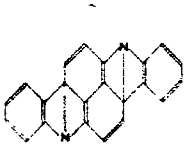

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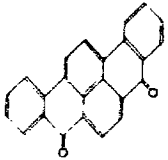

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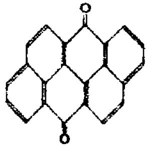

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(*i*) *Dibenzpyrene quinones*

The carcinogenic hydrocarbon 3:4:8:9-dibenzpyrene (see Bachmann *et al.* 1937; Badger *et al.* 1940) had previously been found inhibitory, and an extended investigation has been made of the 5:10-quinone and related compounds. This quinone is also active, and activity is still retained in the corresponding leuco-disulphuric acid ester. The activity of 3:4:8:9-dibenzpyrene and its quinone provides an interesting contrast with the complete inactivity of the non-carcinogenic anthanthrene (Haddow and Robinson 1939) and anthanthrone.

(*j*) *Azo compounds and related products*

In a recent investigation by Cook, Hewett, Kennaway and Kennaway (1940) the primary object was to seek for any evidence of the production of cancer of the bladder by pure chemical compounds which might throw light upon the occupational incidence of this form of cancer in chemical workers, and especially in those handling β-naphthylamine. In this work the three azonaphthalenes were selected for test, 'as it was conceivable that one or other of these might arise by mild oxidation of samples of β-naphthylamine containing α-naphthylamine, and thus be present in the crude base'. The compounds were administered to mice by application to the skin, by subcutaneous injection and by feeding. No tumours of the bladder were obtained, although peculiar changes in that organ were produced by 1:2′-azonaphthalene. Extensive liver changes (cholangioma, hepatoma) were found in mice treated with 2:2′-azonaphthalene and with the corresponding product of reduction and rearrangement, 2:2′-diamino-1:1′-dinaphthyl.* One sarcoma was obtained following subcutaneous injection of 1:1′-azonaphthalene in thirty mice.

Experiments to determine the inhibitory activity of the three azonaphthalenes and other compounds are summarized in table 11. Somewhat surprisingly, 1:2′-azonaphthalene proved strongly inhibitory, whether given by injection in oil or by mouth in the form of a 10 % dispersion. Activity was retained in the 4-hydroxy and 4-amino derivatives, but not in the case of 4-acetylamino-1:2′-azonaphthalene. The toxicity of 1:2′-azonaphthalene was low, and the test animals remained in exceptionally good condition. Significant but lower activity was shown

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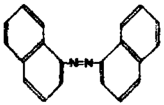
* **2:2′-Diamino-1:1′-dinaphthyl hydrochloride was recently described as exerting strong local anaesthetic action (Supniewski and Gajewska 1939): a dilution of 1:2000 was sufficient to cause complete anaesthesia of the rabbit cornea and the frog skin.**

TABLE 11. AZO COMPOUNDS

experiment number	compound	dose mg.	mean tumour weights (g.) control series	treated series	ratio of means	*t*	*P*
92	1:1′-azonaphthalene	25	24·68	7·79	3·17	9·20	0·001
93	2:2′-azonaphthalene	25	24·68	25·41	0·97	−0·29	0·8
94	2:2′-diamino-1:1′-dinaphthyl	10	20·25	17·51	1·16	0·79	0·5
96	1:2′-azonaphthalene	25	20·25	3·02	6·71	7·58	0·001
97*	1:2′-azonaphthalene	10	3·47	1·79	1·94	3·54	0·01
98	1:2′-azonaphthalene	4 × 50	19·13	6·07	3·15	3·76	0·01
99 M1185	4-hydroxy-1:2′-azonaphthalene	25	26·00	14·90	1·74	4·03	0·001
100	4-amino-1:2′-azonaphthalene	25	17·94	6·72	2·67	5·38	0·001
101 M1752	4-acetylamino-1:2′-azonaphthalene	25	21·54	18·98	1·13	0·74	0·5
102 M1176	1:2′-hydrazonaphthalene	25	20·10	8·27	2·43	2·19	0·05
105	azobenzene	25	24·09	16·82	1·43	1·54	0·2
106 M1058	*p*-dimethylaminoazobenzene ('butter yellow')	25	13·67	8·68	1·57	1·99	0·1
107 M1004B	4′-amino-2:3′-azotoluene ('*o*-aminoazotoluene')	25	9·54	20·17	0·47	−2·47	0·05



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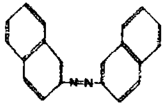

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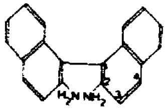

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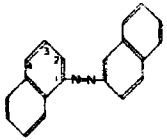

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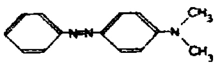

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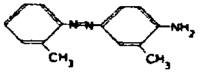

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* Experiment with Crocker mouse sarcoma 180.

by 1:1′-azonaphthalene, and no activity by 2:2′-azonaphthalene. An identical relation was found for the products of reduction and rearrangement of 1:2′- and 2:2′-azonaphthalene.*

The negative results obtained for *o*-aminoazotoluene and *p*-dimethylaminoazobenzene ('butter yellow') are of interest since these isomeric compounds produce cholangioma and hepatoma in rats (Sasaki and Yoshida 1935, Kinosita 1937) as does 2:2′-azonaphthalene in mice.

It was earlier believed that neither *o*-aminoazotoluene (Nakahara and Fujiwara 1937) nor *p*-dimethylaminoazobenzene (Nakahara and Fujiwara 1938) produces tumours locally by injection, and Nakahara and Takahashi (1940) found these compounds to have no growth-inhibitory effect when administered to young rats, although benzpyrene and methylcholanthrene produced such an effect, perceptible but slight. However, more recently Law (1941) reported the induction of fibrosarcomas (35 in 109 mice of the C 57 black strain) following injection of *o*-aminoazotoluene (4′-amino-2:3′-azotoluene), *p*-dimethylaminoazobenzene, 4′-hydroxy-2:3′-azotoluene and 2:3′-azotoluene; only one sarcoma appeared in 106 mice of the Dba strain similarly treated, this in the *p*-dimethylaminoazobenzene series. White (1940) also described a retardation of growth, in young rats, produced by *p*-dimethylaminoazobenzene administered as an addition to a basal diet of relatively low protein content.

In additional experiments on twenty water-soluble azo dyes (table 12), only two instances of significant inhibition were observed. In one case (experiments 123, 124) the effect was not reproduced by a smaller dose, and the other is noteworthy (experiment 118), since the compound is much less soluble than others in the series, and is insoluble in the cold. Cook *et al.* (1940) reported a number of experiments to determine the carcinogenic activity of certain water-soluble azo-dyes (including that tested in experiment 102 of the present series): in their experiments 'not a single tumour appeared that could be attributed to the colouring matters, and such a result is in accord with the view that rapid elimination of these water-soluble sodium sulphonates would tend to prevent their exerting a carcinogenic influence'.

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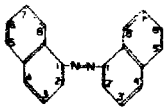
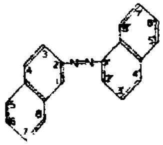
* By analogy with the rearrangement of 1:1′-hydrazonaphthalene, Cook *et al.* (1940) considered 1:2′-diamino-2:1′-dinaphthyl as the probable structure of the diaminodinaphthyl arising from 1:2′-hydrazonaphthalene by rearrangement, although 4:2′-diamino-1:1′-dinaphthyl was not excluded.

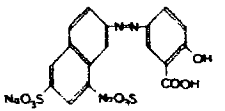
TABLE 12. WATER-SOLUBLE AZO-DYES

experiment number	type  formula A  formula B	dose mg.	mean tumour weights (g.) control series	mean tumour weights (g.) treated series	ratio of means	t	P
108 M 1145	sodium salt of 6:8-disulpho-β-naphthalene-azo-salicylic acid (Colour Index No. 197)	100	**23·09**	**22·15**	1·04	0·16	0·9
109 M 1138	sodium salt of 4-sulpho-α-naphthalene-azo-β-naphthol (Colour Index No. 176) formula A: Substituents SO_3Na (4) OH (2′)	50	**20·93**	**16·14**	1·30	1·36	0·2
110 M 1137	sodium salt of 4-sulpho-α-naphthalene-azo-α-naphthol (Colour Index No. 175) formula A: Substituents SO_3Na (4) OH (4′)	100	**8·11**	**10·80**	0·75	−0·47	0·7
111 M 1141	sodium salt of 4-sulpho-α-naphthalene-azo-β-naphthol-6-sulphonic acid (Colour Index No. 182) formula A: Substituents SO_3Na (4, 6′) OH (2′)	100	**12·43**	**9·00**	1·38	0·76	0·5
112 M 1136	sodium salt of α-naphthalene-azo-β-naphthol-3:6-disulphonic acid (Colour Index No. 88) formula A: Substituents SO_3Na (3′, 6′) OH (2′)	25	**18·57**	**17·62**	1·05	0·20	1·0
113 M 558/2	sodium salt of 4-sulpho-α-naphthalene-azo-β-naphthol-3:6-disulphonic acid (Colour Index No. 184) formula A: Substituents SO_3Na (4, 3′, 6′) OH (2′)	25	**18·57**	**21·24**	0·87	−0·55	0·6
114 M 1147	sodium salt of 4-sulpho-2-hydroxy-α-naphthalene-azo-β-naphthol (Colour Index No. 202) formula A: Substituents SO_3Na (4) OH (2, 2′)	50	**20·93**	**22·11**	0·95	−0·33	0·8

115	M1150	sodium salt of 3:6-disulpho-8-hydroxy-α-naphthalene-azo-phenyl-α-naphthylamine-8-sulphonic acid (Colour Index No. 208) formula A: Substituents SO_3Na (3, 6, 5′) OH (8) —NH— (4′)	100	20·93	24·09	0·87	−0·87	0·4
116	M1149	sodium salt of 4-sulpho-5-nitro-2-hydroxy-α-naphthalene-azo-β-naphthol (Colour Index No. 204) formula A: Substituents SO_3Na (4) NO_2 (5) OH (2, 2′)	100	23·09	11·18	2·07	1·93	0·1
117	M1143	sodium salt of 4-sulpho-α-naphthalene-azo-β-naphthol-6:8-disulphonic acid (Colour Index No. 185) formula A: Substituents SO_3Na (4, 6′, 8′) OH (2′)	100	8·11	5·93	1·37	0·46	0·7
118	M1144	1-sulpho-β-naphthalene-azo-β-naphthol (Colour Index No. 189) formula B: Substituents SO_3H (1) OH (2′)	25	31·93	18·73	1·70	2·98	0·01
119	M1139	sodium salt of 4-sulpho-α-naphthalene-azo-α-naphthol-4-sulphonic acid (Colour Index No. 179) formula B: Substituents SO_3Na (4, 4′) OH (1)	100	12·43	14·25	0·87	−0·39	0·7
120	M1140	sodium salt of 4-sulpho-α-naphthalene-azo-α-naphthol-5-sulphonic acid (Colour Index No. 180) formula B: Substituents SO_3Na (5, 4′) OH (1)	100	12·43	11·10	1·12	0·30	0·8
121	M589/2	sodium salt of 4-sulpho-5-nitro-2-hydroxy-α-naphthalene-azo-α-naphthol (Colour Index No. 203) formula B: Substituents SO_3Na (4′) OH (1, 2′) NO_2 (5′)	25	18·57	13·10	1·42	1·02	0·4
122	M1146	sodium salt of 4-sulpho-2-hydroxy-α-naphthalene-azo-α-naphthol (Colour Index No. 201) formula B: Substituents SO_3Na (4′) OH (1, 2′)	100	31·93	22·07	1·45	2·06	0·1

TABLE 12 (*continued*)

experiment number	type	dose mg.	mean tumour weights (g.)		ratio of means	*t*	*P*
			control series	treated series			
123 M1151	sodium salt of 3-carboxy-4-hydroxy-benzene-azo-α-naphthalene azo-α-naphthol-4-sulphonic acid (Colour Index No. 299)	100	31·93	19·86	1·61	2·28	0·05
124 M1151	sodium salt of 3-carboxy-4-hydroxy-benzene-azo-α-naphthalene azo-α-naphthol-4-sulphonic acid (Colour Index No. 299)	50	8·11	12·28	0·66	−0·72	0·5
125 M1155	sodium salt of 3:6-disulpho-8-hydroxy-α-naphthalene-azo-α-naphthalene-azo-6-phenylamino-1-naphthol-3-sulphonic acid (Colour Index No. 319)	50	20·22	13·14	1·54	1·80	0·1
126 M1152	sodium salt of 3-carboxy-4-hydroxy-benzene-azo-α-naphthalene-azo-1:8-dihydroxynaphthalene-4-sulphonic acid (Colour Index No. 302)	50	13·39	12·82	1·04	0·10	1·0
127 M1153	sodium salt of 5-sulpho-α-naphthalene-azo-α-naphthalene-azo-phenyl-α-naphthylamine-8-sulphonic acid (Colour Index No. 307)	50	13·39	6·77	1·98	1·41	0·2
128 M1154	sodium salt of 6- (or 7-) monosulpho-4-amino-α-naphthalene-azo-α-naphthalene-azo-β-naphthol-6-sulphonic acid (Colour Index No. 316)	100	13·39	11·06	1·21	0·51	0·7



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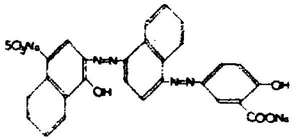

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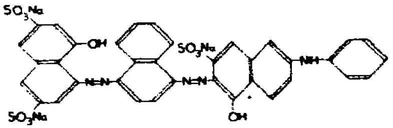

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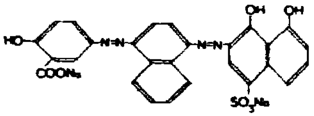

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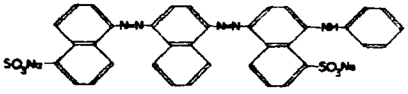

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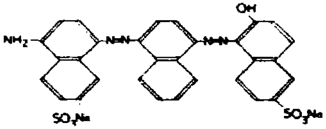

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(*k*) *Compounds exhibiting varied linkage*

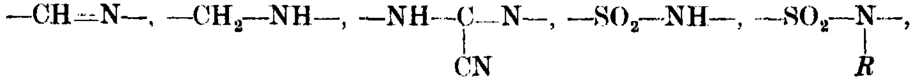


Table 13 presents experiments with compounds in which two naphthalene moieties are united by different types of linkage, viz. —NH—, $-\mathrm{CH{=}N}-$, $-\mathrm{CH_2{-}NH}-$, $-\mathrm{NH}-\underset{\mathrm{CN}}{\underset{|}{\mathrm{C}}}-\mathrm{N}-$, $-\mathrm{SO_2{-}NH}-$, $-\mathrm{SO_2}-\underset{R}{\underset{|}{\mathrm{N}}}-$, and —S—S—. These results are of importance as indicating that certain characters are probably essential in the nature of the bridge between two naphthalene nuclei, if such compounds are to show the inhibitory activity possessed by 1:1′- and 1:2′-azonaphthalene. Significant activity was recorded for 1:1′- and 1:2′-naphthalene-sulphon-naphthylamide but not for the 2:2′ compound, a relationship similar to that obtaining in the azonaphthalenes. It is of considerable additional interest however that the 2:1′ isomeride appeared inactive. No data are as yet available for the carcinogenic activity of these compounds, except in the case of *αα*- and *ββ*-dinaphthylamine, which Boyland and Brues (1937) reported as inactive.

(*l*) *Naphthylamines; naphthaquinone*

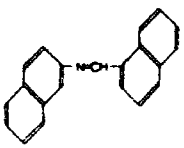
Various considerations led to tests of *β*-naphthylamine and water-soluble sulphonates, naphthylamine sulphonamides, and analogues of vitamin K. No inhibitory activity was noted for any of these compounds in the present experiments (table 14), with the possible exception of one experiment (not reported) in which rats received 2-naphthylamine-4:8-disulphonic acid (in several doses of 100 mg. by intraperitoneal injection) and in which a profound interference with body growth was observed. This was later found to be secondary to an acute atrophic change in the liver, which slowly recovered by normal regeneration. As a side-action, general anaesthesia, followed by complete recovery, was noted after the intraperitoneal injection of *β*-naphthylamine and acetyl *β*-naphthylamine in 25 mg. doses. The effect was not observed with other compounds in the present work, but is probably similar to the anaesthetic action of various steroids recently described by Selye (1941). (Compare also the local anaesthetic action of the hydrochloride of 2:2′-diamino-1:1′-dinaphthyl, p. 271, footnote.)

(*m*) *Arsenonaphthalenes and other arsenical compounds*

The positive results obtained for 1:1′- and 1:2′-azonaphthalene suggested the examination of the three arsenonaphthalenes, as well as organic arsenicals having structural similarity to the polycyclic aromatic hydrocarbons. Only preliminary experiments have been carried out thus far (table 15). 1:1′- and 2:2′-arsenonaphthalenes were administered in a single

TABLE 13. COMPOUNDS EXHIBITING VARIED LINKAGE

experiment number	compound	dose mg.	mean tumour weights (g.) control series	treated series	ratio of means	*t*	*P*
129 M1194	*αα′*-dinaphthylamine	25	24·13	21·91	1·10	0·38	0·8
130 M1195	*ββ′*-dinaphthylamine	25	16·85	20·67	0·82	−1·04	0·4
131 M1199	*α*-naphthaldehyde-*β*-naphthanil	25	16·51	13·76	1·20	0·59	0·6
132 M1198	*α*-naphthaldehyde-*α*-naphthanil	25	16·85	11·40	1·48	1·61	0·2
133 M1201	N-*β*-naphthyl-*α*-naphthylmethylamine	25	16·51	13·68	1·21	0·60	0·6
134 M1199	N-*α*-naphthyl-*α*-naphthylmethylamine	25	16·51	13·26	1·25	0·65	0·6
135 M1294	*α*-(2-naphthylimino)-*α*-(2-naphthylamino) acetonitrile	25	13·58	25·68	0·53	−1·74	0·1
136 M1169	*α*-naphthalene-sulphon-8-aminoquinoline	25	26·68	21·76	1·23	1·10	0·3
137 M1105	1:2′-naphthalene-sulphon-naphthylamide	25	14·49	4·99	2·90	2·49	0·02
138 M1106	2:1′-naphthalene-sulphon-naphthylamide	25	20·91	15·02	1·39	1·06	0·3
139 M1104	1:1′-naphthalene-sulphon-naphthylamide	25	14·49	4·20	3·45	2·70	0·02
140 M1107	2:2′-naphthalene-sulphon-naphthylamide	25	14·49	11·23	1·29	0·77	0·5
141 M1178	1:2′-naphthalene-sulphon-N-methylnaphthylamide	25	26·68	25·83	1·03	0·21	0·9
142 M1179	1:2′-naphthalene-sulphon-N-ethylnaphthylamide	25	26·68	26·79	1·00	−0·03	1·0
143 M1184	1:2′-naphthalene-sulphon-N-*n*-propyl-naphthylamide	25	26·68	22·92	1·16	0·92	0·4
144 M1071	1:1′-dinaphthyldisulphide	25	17·22	16·96	1·02	0·08	1·0



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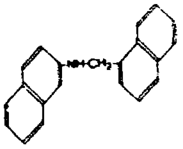

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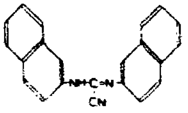

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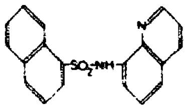

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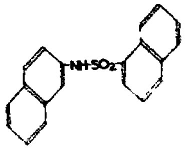

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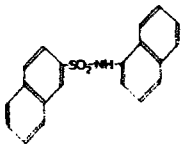

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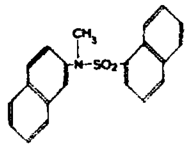

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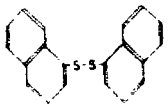

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TABLE 14. NAPHTHYLAMINES; NAPHTHAQUINONE

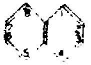
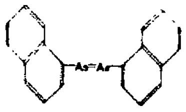
experiment number	type 	dose mg.	mean tumour weights (g.) control series	mean tumour weights (g.) treated series	ratio of means	*t*	*P*
145	β-naphthylamine	25	15·17	16·57	0·92	−0·31	0·8
146	acetyl-β-naphthylamine	25	21·96	20·77	1·06	0·21	0·9
147	2-naphthylamine-1-sulphonic acid	100	17·73	19·05	0·93	−0·31	0·8
148	2-naphthylamine-6-sulphonic acid	100	17·73	15·64	1·13	0·54	0·6
149	2-naphthylamine-6:8-disulphonic acid	100	17·73	19·01	0·93	−0·34	0·8
150	2-naphthylamine-4:8-disulphonic acid	5 × 50	8·35	11·28	0·74	−0·70	0·5
151	2-naphthylamine-4:8-disulphonic acid	100	17·73	19·53	0·91	−0·40	0·7
152	2-naphthylamine-5:7-disulphonic acid	100	17·73	21·66	0·82	−1·04	0·4
153	2-naphthylamine-4:8-disulphonamide	25	20·22	13·39	1·51	1·60	0·2
154	1-naphthylamine-5-sulphonamide	25	17·22	19·00	0·91	−0·61	0·6
155	1-naphthylamine-7-sulphonic acid	25	17·22	20·95	0·82	−1·26	0·3
156	1:4-naphthaquinone	0·5	8·04	14·78	0·54	−1·70	0·2
157	2-methyl-1:4-naphthaquinone	5	8·04	12·69	0·63	−1·33	0·2
158	2-methyl-1:4-naphthahydroquinone diacetate	25	8·04	6·57	1·22	0·45	0·7

TABLE 15. ARSENONAPHTHALENES AND OTHER ARSENICAL COMPOUNDS

experiment number	compound	dose mg.	mean tumour weights (g.) control series	mean tumour weights (g.) treated series	ratio of means	*t*	*P*
159	1:1′-arsenonaphthalene	1	11·28	13·31	0·85	−0·53	0·6
160	1:1′-arsenonaphthalene	1	27·65	17·08	1·62	2·93	0·01
161	2:2′-arsenonaphthalene	1	11·28	11·06	1·02	0·05	1·0
162	2:2′-arsenonaphthalene	1	27·65	19·59	1·41	2·27	0·05
163	1:2:8:9-dibenzphenarsazinic acid (sodium salt)	2	19·92	15·79	1·26	0·85	0·5
164	1:2:8:9-dibenzphenarsazinic acid (sodium salt)	7 × 1	21·89	19·59	1·12	0·42	0·7
165	1:2:8:9-dibenz-10-chloro-5:10-dihydrophenarsazine	20	10·45	8·89	1·18	0·54	0·6



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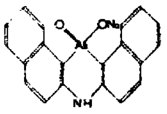

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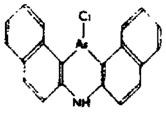

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dose approaching the maximum tolerated. In two experiments with these compounds (159, 161) no inhibition was observed, although a striking effect was noted in a large reduction of food intake. In experiments 160 and 162, on the other hand, the same doses produced manifest toxic effects (possibly on account of the fact that the compounds were less completely dissolved): in this case it is felt that the recorded inhibition had no real importance. It is desirable to test the activity of 1:2′-arsenonaphthalene, and this work is being continued.

(*n*) *Derivatives of triphenylethylene*

Following the statement of Zondek (1936*a*) that prolonged treatment with folliculin produces considerable retardation of body growth, numerous papers have testified to the capacity of oestrogenic substances, both naturally occurring and synthetic, to inhibit growth (see discussion). Table 16 presents the results obtained in tests with six derivatives of triphenylethylene, the oestrogenic activity of which was described by Robson and Schönberg (1937). These compounds are possibly of special significance since they, with triphenylethylene itself, may be formulated to indicate relationship to a *meso*- (and 4-) substituted benzanthracene with two rings disrupted. In certain of these experiments an inhibitory effect on body growth was detectable, and at autopsy the regressive change in the testis, which is well recognized as a result of oestrogen action, was also apparent. Table 16 includes the relevant data from experiment 170. In this case the evidence for inhibition of body growth is only presumptive (although reasonable since the batches were carefully balanced), as the weights of the rats at the beginning of the experiment were not recorded.

(*o*) *Diphenyl derivatives of indene, β-naphthindole and β-naphthofuran*

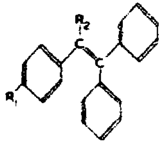
Tests with four derivatives of indene, β-naphthindole and β-naphthofuran, which possess a certain degree of relationship to stilbene and triphenylethylene, and are with one exception oestrogenic, are given in table 17. Inhibition to a significant extent was observed for 1:2-diphenylindene only.

(*p*) *Cholic acid; deoxycholic acid*

Negative results were obtained with deoxycholic and cholic acids (3:12-dihydroxy and 3:7:12-trihydroxy cholanic acid respectively) (table 18). Although deoxycholic acid is an important constituent of normal bile, injection of the compound has recently been shown (Cook, Kennaway and Kennaway 1940; confirming Ghiron 1939) to result in the

TABLE 16. DERIVATIVES OF TRIPHENYLETHYLENE

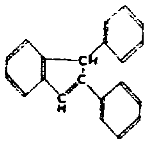
type



experiment number	R_1	R_2		dose mg.	mean tumour weights (g.) control series	mean tumour weights (g.) treated series	Ratio of means	t	P	oestrogenic activity determined in ovariectomized mice: dose mg./20 g.	activity: mice in full oestrus	activity: duration to half action (days)
166 M612		CH_3	triphenyl methylethylene	25	10·45	3·03	3·45	2·89	0·01	0·02	6/6	8
167 M605		CN	triphenyl cyanoethylene	25	27·86	17·31	1·61	4·11	0·001	0·02 1·00	1/9 10/10	4 12
168 M815		Cl	triphenyl chloroethylene	25	27·86	16·91	1·65	4·56	0·001	0·01 0·02	4/10 10/10	4 27
169 M604		$CO.NH_2$	triphenyl-acrylic amide	25	12·57	12·23	1·03	0·10	1·0	—	—	—
					tumour weights						mice in full oestrus	duration to full oestrus
170 M842	CH_3	Cl	αα-diphenyl-β-p-tolyl-β-chloroethylene	25	22·66	16·05	1·41	2·06	0·1	0·1	3/8	ca. 2
					body weight							
					105·5	93·1	1·13	2·27	0·05	—	—	—
					testis weight							
					0·99	0·33	2·98	—	0·001	—	—	—
171 M852	CH_3	CH_3	αα-diphenyl-β-methyl-β-p-tolylethylene	25	10·45	6·99	1·49	1·26	0·3	0·1	0/8	—

Table 17. Diphenyl derivatives of indene, β-naphthindole and β-naphthofuran

experiment number	compound	dose mg.	mean tumour weights (g.)		Ratio of means	t	P	oestrogenic activity determined in ovariectomized mice		
			control series	treated series				dose mg./20 g.	activity: mice in full oestrus	activity: duration of full oestrus (days)
172 M622	1:2-diphenylindene	25	10·45	4·62	2·26	2·14	0·05	0·1	0/6	—
								20·0	6/6	15
173 M623	2:3-diphenylindene	25	12·57	14·36	0·88	−0·48	0·7	0·1	0/6	—
								20·0	6/6	14
174 M610	2:3-diphenyl-β-naphthindole	25	22·66	19·58	1·16	1·03	0·4	—	—	—
175 M607	2:3-diphenyl-β-naphthofuran	25	27·86	23·02	1·2	1·71	0·2	10·0	0/3	—
								5·0	0/3	—
								20·0	3/3	2



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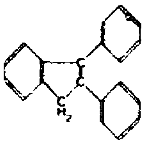

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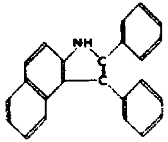

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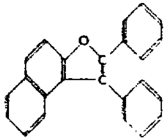

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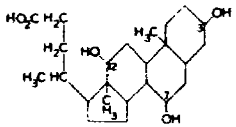

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Table 18. Cholic acid; deoxycholic acid

experiment number	compound	dose mg.	mean tumour weights (g.) control series	mean tumour weights (g.) treated series	ratio of means	t	P
176	cholic acid (3:7:12-trihydroxycholanic acid)	25	21·96	26·60	0·83	−0·95	0·4
177	deoxycholic acid (3:12-dihydroxycholanic acid)	25	15·35	18·57	0·83	−0·56	0·6

production of sarcoma, both in stock mice (4 sarc./10 mice/422 days) and mice of the C3H strain (7 sarc./10 mice/207 days); only one epithelioma appeared on repeated and prolonged application of the acid to the skin (in 100 mice). Dehydrogenation of dehydronorcholene (non-inhibitory, Haddow and Robinson 1937), which may be obtained from deoxycholic acid by the procedure of Wieland, yields the benzanthracene hydrocarbon methylcholanthrene (Wieland and Dane 1933; Cook and Haslewood 1933); again, Fieser and Newman (1935) showed that methylcholanthrene could also be obtained from cholic acid. It will therefore be important to determine, if possible, whether deoxycholic acid possesses intrinsic carcinogenic activity, or is subject to a transformation in vivo yielding traces of an active product. In discussing this question Cook *et al.* make the following comment: 'We have no evidence that deoxycholic acid can be converted in vivo into methylcholanthrene, but...the slow action and large amount required of the acid, and the superiority of injection over external application, are compatible with conversion into some more active compound.'

(*q*) *Styryl* 430, *isomers and analogues*

Styryl 430, which Browning, Gulbransen and Niven (1936) found capable of producing sarcomata in mice, had previously been tested for inhibitory properties but with irregular results. Further experiments with this compound, and various isomers and analogues, are given in table 19.

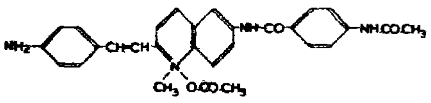
(*r*) *Miscellaneous compounds*

Of the miscellaneous group of compounds in table 20, attention may be drawn to those giving indications of significant inhibitory activity: 5-amino-1:2-benzanthracene, 4-methylbenzanthrone, benzanthrone-4-aldehyde, di-α-naphthyl ether and 1:1′-dinaphthylene-2:2′-oxide. Among these compounds, 5-amino-1:2-benzanthracene is under test for carcinogenicity, and has produced one spindle-cell sarcoma (day 206).

Of compounds yielding negative results 3(N)-4-pyridinopyrene (Vollmann, Becker, Corell and Streeck 1937), a nitrogenous analogue of 3:4-benzpyrene, has proved non-carcinogenic (0 pap., 0 epith./19 mice/748 days, Badger *et al.* 1940). 1:1′-Dinaphtho-2:2′-orthodiazine was tested following the results obtained with the azonaphthalenes, although it is more nearly analogous to the non-carcinogenic 3:4:5:6-dibenzphenanthrene: in a continuation of these experiments it is hoped to examine 2:2′-dinaphtho-1:1′-orthodiazine and 1:2′-dinaphtho-2:1′-orthodiazine, which may be regarded as similarly related to 1:2:7:8-dibenzphenanthrene (picene) and 1:2:5:6-dibenzphenanthrene, respectively.

TABLE 19. STYRYL 430, ISOMERS AND ANALOGUES

experiment number		compound	dose mg.	mean tumour weights (g.) control series	mean tumour weights (g.) treated series	ratio of means	t	P
178	M 600	2-(*p*-aminostyryl)-6-(*p*-acetylaminobenzoylamino) quinoline methoacetate. 'Styryl 430'	25	11·51	7·34	1·57	0·93	0·4
179	M 600	2-(*p*-aminostyryl)-6-(*p*-acetylaminobenzoylamino) quinoline methoacetate. 'Styryl 430'	25	26·41	23·36	1·13	0·80	0·5
180	M 629	2-(*p*-aminostyryl)-6-(*m*-acetylaminobenzoylamino) quinoline methoacetate	25	19·33	7·17	2·70	2·57	0·02
181	M 634	2-(*p*-acetylaminostyryl)-6-(*p*-aminobenzoylamino) quinoline methoacetate. 'Cohen 245'	25	21·22	23·38	0·91	−0·45	0·7
182	M 106	2(4′-β-hydroxyethylamino-3′-methylstyryl)-6-*n*-caproylamino-quinoline methosulphate. 'Styryl 106'	25	21·60	12·59	1·72	1·43	0·2
183	M 207	2-(*p*-acetylaminostyryl)-6-β-phthalimidoethylamino-quinoline methoacetate	25	21·22	19·78	1·07	0·41	0·7
184	M 632	2-styryl-6-(*p*-acetylaminobenzoylamino) quinoline methoacetate	25	21·22	24·78	0·86	−0·88	0·4
185	M 633	2-(*p*-acetylaminostyryl)-6-benzoylamino quinoline methoacetate	25	9·54	16·33	0·58	−1·44	0·2
186	M 627	2-(*p*-aminostyryl)-6-acetylaminoquinoline methoacetate	25	19·33	5·22	3·70	3·20	0·01
187	M 206	2-(*p*-aminostyryl)-6-benzoylamino quinoline methoacetate	25	11·51	3·53	3·26	2·02	0·1
188	M 625	6-(*p*-acetylaminobenzoylamino) quinaldine methochloride	10	19·33	17·78	1·09	0·27	0·8
189	M 630	2-(*p*-aminostyryl)-β-naphthoquinoline methoacetate	25	21·22	26·02	0·82	−1·34	0·2
190	M 631	2-(*p*-acetylaminostyryl)-β-naphthoquinoline methoacetate	25	9·54	12·30	0·78	−0·59	0·6



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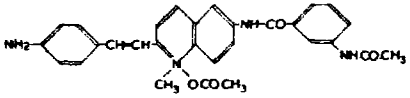

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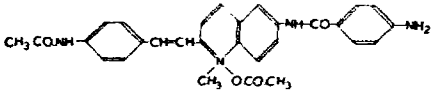

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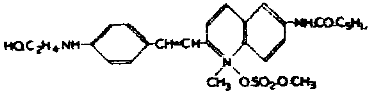

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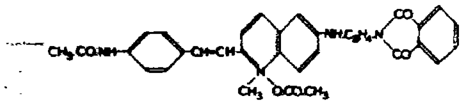

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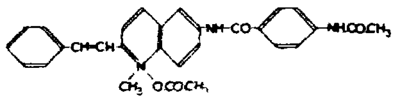

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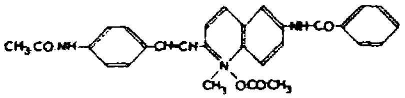

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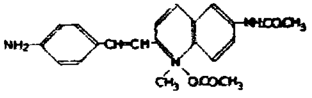

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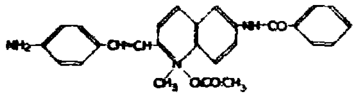

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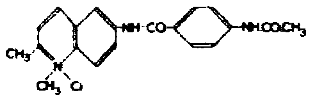

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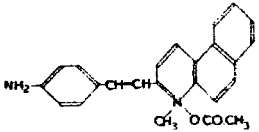

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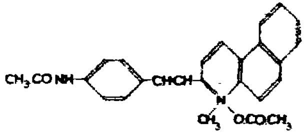

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Some comment is necessary on the results obtained with heptaldehyde and citral. Strong (1938, 1939) showed that the growth of spontaneous mammary tumours in mice is inhibited by the oral administration of heptaldehyde. This observation was confirmed by Boyland and Mawson (1938; see also Boyland 1940), who showed in addition that citral had a greater inhibitory effect. The results in the present tests show no effect on the 15th day following a single intraperitoneal injection in oil, but a strong inhibitory effect was obvious by inspection at an earlier stage (8–10 days), passing off with some rapidity in the last few days of the experiment.

4. Discussion

(a) *Correlation of inhibitory activity and carcinogenicity*

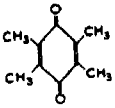
(i) *The carcinogenic hydrocarbons and related compounds.*

It has already been stated that doses employed in these experiments were in most cases only 50 % of those in earlier work, since the main object was the selection of more active compounds. That a single dose of *c*. 25 mg./100 g. is probably insufficient to produce a demonstrable effect in the case of weaker compounds is suggested by figure 1: although all seven of the 5-alkyl benzanthracenes tested produce epitheliomata, statistically significant inhibition was detected only in four members, which included the first three of the series. Yet the values taken together indicate a general trend, and it is justifiable to conclude that increase in size of the substituent is accompanied by a decline both in inhibitory activity and the power to induce epithelioma in mice. The correspondence is possibly even closer when carcinogenicity is measured by the capacity to evoke sarcomata. These data have not been presented graphically since they are derived from different sources and are incomplete (for the 5-ethyl and 5-*n*-propyl members). However, figures given by Fieser (1938) show a carcinogenic index of 46 for 5-methyl-1:2-benzanthracene when tested in strain A mice, and the value is zero for the 5-*n*-butyl, -*n*-amyl, -*n*-hexyl and -*n*-heptyl derivatives in stock mice (Badger *et al.* 1940).

A similar relation probably holds for the 10-alkyl benzanthracenes, although the rate of decline is steeper: thus while 10-methyl-1:2-benzanthracene is actively carcinogenic and inhibitory, neither activity is present in the 10-*iso*propyl compound. Data on the sarcoma-producing capacity of 10-substituted benzanthracenes in strain A mice are also provided by Fieser (1938). On the basis of these figures the carcinogenic index for 10-methyl-1:2-benzanthracene is 89, for 10-ethyl-1:2-benzanthracene 24, and zero for the 10-*n*-propyl, -*iso*propyl, -allyl, -*n*-butyl and -*n*-amyl derivatives.

TABLE 20. MISCELLANEOUS COMPOUNDS

experiment number		compound	dose mg.	mean tumour weights (g.) control series	treated series	Ratio of means	*t*	*P*
191	M1552	duroquinone	20	15·57	23·48	0·67	−3·11	0·01
192	M1601	benzil	25	13·75	13·45	1·02	0·08	1·0
193	M1047	'napthasultam'	25	16·65	17·43	0·96	−0·14	0·9
194	M572	2-mercaptobenzthiazole	25	19·95	21·00	0·95	−0·27	0·8
195	M573	2-phenylbenzthiazole	25	19·95	22·93	0·87	−0·69	0·5
196		octamethyl-diphenyl methane	25	15·35	13·25	1·16	0·38	0·8
197	M1192	$\alpha\alpha'$-dinaphthol	25	24·13	29·53	0·82	−1·24	0·3
198	M1193	$\beta\beta'$-dinaphthol	25	24·13	18·83	1·28	1·15	0·3
199	M1196	di-β-naphthyl ether	25	26·00	15·98	1·63	3·36	0·01
200	M1249	di-α-naphthyl mercury	4·3	13·67	16·52	0·83	−1·09	0·3
201	M1068	2:2'-dihydroxy-1:1'-dinaphthyl methane	25	15·39	22·50	0·68	−1·58	0·2
202	M1197	1:1'-dinaphthylene-2:2'-oxide	25	29·24	14·89	1·96	2·34	0·05
203		α-naphthil	20	20·56	17·13	1·20	0·80	0·5
204		β-naphthil	25	20·56	13·44	1·53	1·71	0·1
205		2:2'-dinaphthyl ethane	25	27·86	25·70	1·08	0·82	0·5
206	M38	quindoline methochloride	1	15·87	16·00	0·99	−0·03	1·0
208		5-amino-1:2-benzanthracene	25	29·60	18·66	1·59	2·18	0·05
210	M1190	4-methylbenzanthrone	25	26·00	12·86	2·02	4·49	0·001
211	M1191	benzanthrone-4-aldehyde	25	26·00	15·58	1·67	4·17	0·001
212		3(N)-4-pyridinopyrene	25	20·23	17·21	1·18	1·04	0·4
213		1:1'-dinaphtho-2:2'-orthodiazine	25	15·35	19·10	0·80	−0·67	0·6
214	M1078	4-amino-3':4'-phthaloyl-thiozanthone	25	16·85	20·69	0·81	−1·01	0·4
215	M1248	1:2-benz-5:6-phthaloyl-acridine	25	29·24	23·98	1·22	0·88	0·4
216	M1077	'soledon blue', 2*RC*	25	16·85	25·77	0·65	−1·39	0·2
217	M1499	dehydro-14-phenyl-1':2':8':9'-dibenzoxanthenium chloride	1	20·56	11·25	1·83	2·04	0·1
218	M980	pyranthrone	25	20·71	22·25	0·93	−0·57	0·6
219	M1014	flavanthrone	25	22·24	24·53	0·91	−0·73	0·5
220	M1074	allomesonaphthodianthrone	25	16·65	18·09	0·92	−0·35	0·8
223	M1012	dibenzanthrone	25	24·68	17·10	1·44	1·50	0·2
224	M1013	*iso*-dibenzanthrone	25	24·68	20·91	1·18	0·88	0·4
226		heptaldehyde (oenanthal, *n*-heptoic aldehyde)	1·5 c.c. of 1 in 7 sol. in oil	18·82	10·99	1·71	1·39	0·2
227		citral	0·5 c.c. of 1 in 7 sol. in oil	18·82	15·48	1·22	0·48	0·7



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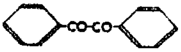

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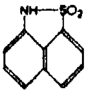

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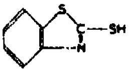

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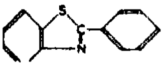

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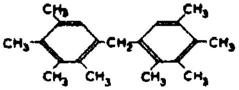

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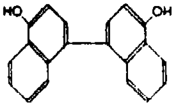

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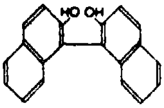

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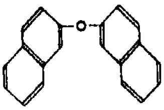

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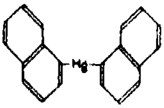

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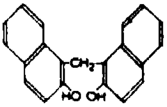

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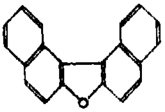

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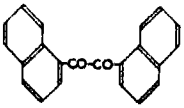

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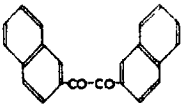

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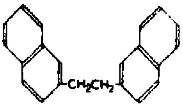

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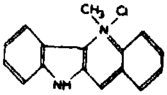

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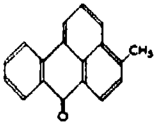

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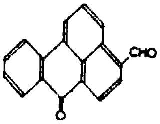

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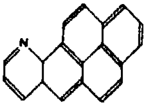

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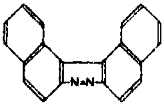

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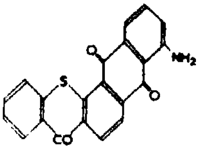

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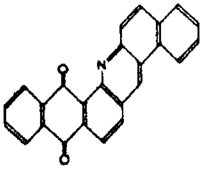

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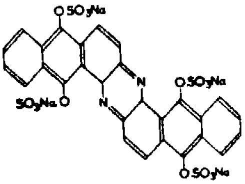

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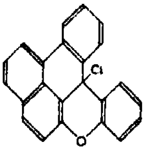

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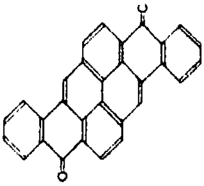

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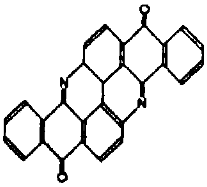

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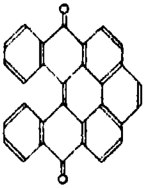

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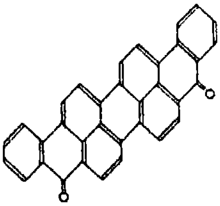

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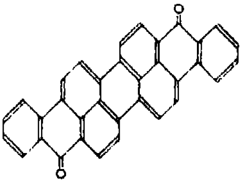

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A striking degree of correspondence is often shown by the properties of closely related compounds. Thus while the carcinogenic hydrocarbon 1:2:5:6-dibenzfluorene is strongly inhibitory, 3:4:5:6-dibenzfluorene is inactive in both respects. Again, 1:2:3:4-tetramethylphenanthrene appears non-inhibitory, but 1:2-dimethylchrysene and 1:2:3:4-dibenzphenanthrene exhibit pronounced inhibitory and carcinogenic properties. However, although exceptionally low dosage must be taken into account, no inhibition was demonstrated for 10-β-hydroxyethyl-, 10-acetoxymethyl-, 9:10-bishydroxymethyl-, 9:10-bisacetoxymethyl- or 9-methyl-10-cyano-1:2-benzanthracene, all of which are carcinogenic.

In the course of these investigations it has become apparent that carcinogenicity is not an absolute property but one influenced in its manifestation by the varied sensitivity of different tissues, strains and species. Other factors may operate in particular cases, including the possibility that certain compounds, inactive of themselves, may undergo transformation to an active compound in vivo (e.g. vide 2:2′-azonaphthalene infra). This has already been considered for deoxycholic acid, and it had previously been suggested by Cook (1939) that the slight carcinogenic activity of certain dihydro compounds might be related to their dehydrogenation to the aromatic structure. It is obvious that were such transformation to occur slowly, no evidence of inhibition would be obtained in a short experiment.

In addition to instances in which compounds have proved carcinogenic although no inhibitory effect has been detected, examples have been encountered of compounds possessing inhibitory activity but appearing non-carcinogenic. Three such compounds, previously reported, later proved to be carcinogenic, in experiments finally reported by Badger *et al.* (1940), and a proportion of the exceptions in this category may be accounted for in the same manner: 'The experiments of Haddow and Robinson...on the correlation between growth-inhibitory action and carcinogenic activity of polycyclic compounds had shown that the 3- and 7-methyl derivatives of 1:2-benzanthracene were anomalous inasmuch as they produced strong inhibition of growth, although our earlier tests had not given tumours when the compounds were applied to the skin of mice. The experiments now reported on the marked sarcoma-producing power of these two hydrocarbons remove this discrepancy. Similar considerations apply in the case of "acenaphthanthracene"; this hydrocarbon, which also gave negative results in earlier tests for carcinogenic action, showed marked inhibition of tumour growth. In more extensive tests the compound has given ten tumours...on application to the skin of fifty mice, and has also given sarcomas in six out of ten mice into which it was injected subcutaneously.'

Even when full allowance has been made for cases explicable as described, there still remains a number of compounds which are strongly inhibitory but yield few or no tumours in exhaustive tests. An outstanding member of this class appears to be 1:2′-azonaphthalene. In addition there must be considered a group of growth-inhibitory oestrogenic substances which, although not carcinogenic in the usual sense, nevertheless are clearly associated with the induction of individual types of tumour under special conditions. These classes are of sufficient importance to warrant separate discussion. Meanwhile attention can be drawn to two conditions in which a substance, not demonstrably carcinogenic, may show inhibitory properties similar to those of the carcinogenic compounds, viz.:

(1) if the cells become continuously adapted to its action, without discontinuous change involving an alteration of growth rate, and

(2) if for an unknown reason the induction of a discontinuous adaptation is difficult or impossible.

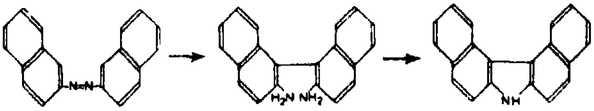
In illustration of the first postulate may be mentioned the description by Selye (1940) of cellular adaptation to oestrogen overdosage (acquired hormone resistance). The second finds an accurate parallel in the varying facility with which different trypanocides induce the formation of drug-resistant protozoal strains.

(ii) *Azonaphthalenes.*

At the moment, interpretation of the results obtained with the azonaphthalenes is difficult, and the most marked contrast is presented by the behaviour of the 1:2′ and 2:2′ compounds. Cook *et al.* (1940) pointed out that azo substances may be reduced in the body to amines by way of the hydrazo compound. When liver changes were found in mice treated with 2:2′-azonaphthalene, the fact that the hydrazonaphthalenes are susceptible to a benzidine type of rearrangement led these workers to make tests with the compound which might thus be formed, viz. 2:2′-diamino-1:1′-dinaphthyl. This compound induced liver changes with even greater facility than 2:2′-azonaphthalene, and the result was specially significant in view of the ease with which the diamino-dinaphthyl undergoes deamination to 3:4:5:6-dibenzcarbazole, which Boyland and Brues (1937) had already shown to be capable of producing liver tumours, epithelioma and sarcoma in mice. The relations between these compounds, with their inhibitory and carcinogenic activity, are shown in table 21.

Taken alone, these relations form a consistent scheme, compatible with and even supporting the suggestion that 2:2′-azonaphthalene is not inherently carcinogenic but acts by a transformation to 3:4:5:6-dibenzcarbazole. Conversion in the liver might account for the localization of tumour production to that organ, and the fact that the intermediate diamine is even more potent than the azo compound is also consistent. However, no facts are as yet available regarding the changes undergone by 2:2′-azonaphthalene in vivo, although experiments are being carried out in this Institute by Mr F. L. Warren and will be reported later.

TABLE 21



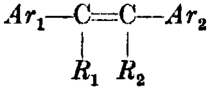
	2:2′-azonaphthalene	2:2′-diamino-1:1′-dinaphthyl	3:4:5:6-dibenzcarbazole
inhibitory activity	−	−	+
tumour production	cholangioma hepatoma	cholangioma hepatoma	cholangioma hepatoma epithelioma sarcoma

(iii) *Oestrogenic compounds.*

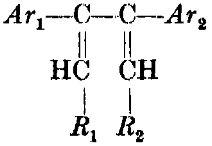
Interest in the relation between carcinogenic and oestrogenic activity was stimulated by the work of Cook and Dodds (1933) and Cook, Dodds, Hewett and Lawson (1934). These authors found a degree of oestrogenic activity in the carcinogenic hydrocarbons 5:6-*cyclo*penteno-1:2-benzanthracene and 3:4-benzpyrene, and proceeded to examine compounds containing the same ring system as the carcinogenic hydrocarbons, but with polar groups present and with some rings reduced. 1:2:5:6-Dibenzanthracene itself proved non-oestrogenic, but high activity was shown by the 9:10-diethyl, di-*n*-propyl and di-*n*-butyl derivatives of 1:2:5:6-dibenz-9:10-dihydroanthraquinol, maximal potency being obtained with the di-*n*-propyl compound. This substance was later found by Haddow and Robinson (1937) to be strongly inhibitory, and still later reported as having produced a few sarcomata in rats (Bachmann and Bradbury 1937).

The work of Cook and Dodds subsequently led to a considerable investigation of the relations between molecular structure and oestrogenic activity, special attention being paid to various hydrocarbon derivatives of ethylene (Dodds and Lawson 1938). Of the oestrogenic compounds examined for growth-inhibitory activity in the present work, the active analogues of triphenylethylene are of special interest as possessing a remote degree of similarity to a *meso*-substituted benzanthracene. Reports by various workers of growth-inhibitory effects produced by diethylstilboestrol (vide infra) suggested an extension of the present investigation by an examination of certain naphthalene and other analogues of stilbene and its derivatives, as well as similarly substituted ethanes and acetylenes, of the general types:

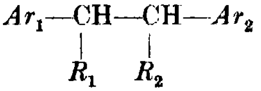
$$Ar_1\text{—C}\equiv\text{C—}Ar_2$$



$$Ar_1\text{—C}(R_1)\text{=C}(R_2)\text{—}Ar_2$$



$$Ar_1\text{—C}(\text{=CH}R_1)\text{—C}(\text{=CH}R_2)\text{—}Ar_2$$



$$Ar_1\text{—CH}(R_1)\text{—CH}(R_2)\text{—}Ar_2$$

(Ar_1 = phenyl, α-naphthyl, β-naphthyl. $Ar_2 = \alpha$-naphthyl. R_1, R_2 = Me, Et.)*

For several of the proposed ethylene structures the relationship to known inhibitory polycyclic hydrocarbons (e.g. 1:2:5:6-dibenzanthracene, 1:2:5:6-dibenzfluorene) is much closer than for the triphenylethylene derivatives already tested. These experiments are not sufficiently advanced to permit any conclusions to be drawn: it may be mentioned, however, that little or no inhibitory activity has been detected for 2:2′- and 1:2′-dinaphthyl ethylene.

It has been widely accepted that oestrogens are to be regarded as growth-promoting agents (e.g. Allen 1935; Doisy 1941). There is certainly ample evidence for this belief, and much more than obtains in the case of the carcinogenic hydrocarbons. But evidence is also accumulating to show that oestrogens are capable of marked inhibitory effects, in many cases of the same nature as those produced by carcinogenic polycyclic hydrocarbons. The oestrogens thus provide a second example of an apparently paradoxical relation, in that compounds possessing growth-inhibitory properties may also be associated with physiological stimulation of growth and with the induction of tumours in special cases.

The older literature contains several references suggesting retardation of growth by oestrogens. More recently Zondek (1936*a*) described inhibition of body growth by administration of oestrin, and similar results were

* See Badger 1941.

reported by Noble (1939*a*) and Gaarenstroom and Levie (1939) in the case of diethylstilboestrol. These effects appear similar to the retardation of body growth produced by intraperitoneal injection of many carcinogenic hydrocarbons (Haddow *et al.* 1937).

Many other papers have reported a more specific inhibitory effect of oestrogens, viz. the production of sterility, azoospermia or testicular atrophy (e.g. Burrows and Kennaway 1934; Lacassagne 1934; Pincus and Kirsch 1936). This phenomenon also finds a parallel in the action of many carcinogenic hydrocarbons (Haddow and Robinson 1937): 'apart from their action on body growth and tumour growth the carcinogenic hydrocarbons produced other effects which are no doubt correlated....The fertility of the treated animals was very definitely lowered, and in many cases the ovary showed suppression of ovulation and the testis a variable reduction of spermatogenesis....' The extent of this effect, whether produced by carcinogens or oestrogens, can readily be estimated from the testis weights in treated animals as compared with controls.

Other effects more recently described are the induction of benignancy in the mouse sarcoma 180 (Salter, Nathanson and Wilson 1941), and the inhibition of hair growth (Gardner and De Vita 1940) and of mammary growth (Gardner 1941) by large amounts of oestrogen.

Such results are of significance in their bearing on the theory of carcinogenesis by cellular inhibition. The induction of tumours by oestrogens is most easily elicited in tissues which are highly responsive to the physiological action of these compounds. The largest number of reports deals with the production of mammary cancer* in mice of susceptible strains, and recent papers record this effect as produced by the synthetic oestrogens diethylstilboestrol (Lacassagne 1938; Shimkin and Grady 1940) and triphenylethylene (Robson and Bonser 1938).

Since the testis is markedly sensitive to the inhibitory action of oestrogens, the production of testicular tumours and hypertrophy of the interstitial cells, in the testes of mice receiving oestrogens, is of special significance (Gardner 1937; Hooker, Gardner and Pfeiffer 1940). Bonser and Robson (1940) found advanced atrophy, with arrest of spermatogenesis, in the testes of RIII and CBA males treated with triphenylethylene or oestradiol dipropionate: in Strong A males treated with triphenylethylene the testes showed no gross diminution in size, but interstitial hyperplasia, proceeding to nodule formation which in advanced stages appeared to be malignant, occurred in a large proportion of cases.

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* Malignant and benign mammary tumours may also be induced by similar means in the rat (Geschickter 1939; Noble, McEuen and Collip 1940), and it is of great interest that Noble and Collip (1941) noted regression following removal of the stimulus. Lipschütz and Vargas (1939) also observed that certain benign fibroid tumours, obtained by treatment with oestradiol, regressed when such treatment was discontinued.

While the carcinogenic activity of oestrogens is influenced by their physiological specificity, sarcoma production has also been reported (Gardner, Smith, Strong and Allen 1936 *a*, *b*; Suntzeff, Burns, Moskop and Loeb 1936; Lacassagne 1937, 1939; Bachmann and Bradbury 1937). In painting experiments by Bachmann *et al.* (1937) no tumours were obtained with the dibenzanthracene diols, oestrone, oestriol or other compounds related to the sterols and oestrogenic hormones. These authors emphasized that such negative results merely record a failure to induce tumours of the skin, and have no bearing on the association of oestrogenic substances with other forms of cancer: 'obviously a compound which can produce cancer is entitled to be termed carcinogenic without any qualifying restriction as to the manner in which this is achieved.' Negative results by painting and injection were also reported for triphenylethylene (Badger *et al.* 1940).

(*b*) *Structural requirements for inhibitory and carcinogenic activity*

The relation between molecular structure and biological activity is still insufficiently known to allow accurate prediction in individual cases. But inhibitory power is generally dependent (1) on an optimal degree of molecular complexity, and (2) on certain more specific requirements. These conditions are best exemplified by the activity of five of the fifteen possible hydrocarbons containing systems of five condensed benzene rings (1:2:5:6- and 1:2:7:8-dibenzanthracene, 1:2:3:4- and 1:2:5:6-dibenzphenanthrene, 3:4-benzpyrene), and by closely related derivatives of 1:2-benzanthracene, chrysene and 3:4-benzphenanthrene. Limited molecular simplification may be carried out without loss of activity (e.g. 5-, 9- or 10-methyl-1:2-benzanthracene) and may proceed to a considerable extent (e.g. 1:2:3:4-tetramethylphenanthrene, 9:10-dimethylanthracene) without complete loss of carcinogenicity. Further, the results obtained with derivatives of triphenylethylene suggest that inhibitory activity may still be shown by compounds diverging widely from the polycyclic structure and possessing only a skeletal resemblance.* Retention of activity with increased complexity is exemplified by 3:4:8:9-dibenzpyrene. Restriction may, however, be narrow, since it had previously been found that an enormous loss of inhibitory activity occurs in passing from 5:9:10-trimethyl-1:2-benzanthracene to the 5:6:9:10-tetramethyl derivative.

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* Dodds, Lawson and Williams (1941) have recently observed two spindle-celled skin tumours in fifty stock mice painted twice weekly for over 12 months with a 0·3 % solution of α-ethyl-β-*sec*-butylstilbene in benzene. This compound presents a fairly close relationship to 3:4-benzpyrene, and is weakly oestrogenic (personal communication from Professor E.C. Dodds 1941).

So far as inhibitory activity is concerned in derivatives of 1:2-benzanthracene and its analogues, the reactive ring of the anthracene system may be replaced, without loss of activity, by a five-membered ring, a five-membered ring containing nitrogen, a six-membered ring containing one nitrogen atom, or a six-membered ring containing nitrogen and sulphur. Also, the effects of methyl substitution in 1:2-benzanthracene, 1:2-benzacridine and 1:2- and 3:4-benzphenothiazine appear to follow a similar law. In view of the above, the pronounced activity of 1:1′- and 1:2′-azonaphthalene is of special interest, as is also that shown by the 1:1′- and 1:2′- (but not 2:1′-) naphthalene-sulphon-naphthylamides.

Diminution of inhibitory effect with increased substituent size is shown in the 5-alkyl benzanthracenes, although the same relation does not necessarily obtain for other positions. The influence of the nature of the substituent is seen in the contrast between 10-methyl-, 10-amino- and 10-cyano-1:2-benzanthracene (inhibitory) and 10-*iso*propyl-1:2-benzanthracene (inactive).

Numerous experiments have shown that solubilization of an active compound usually entails decrease or loss of activity, although certain exceptions have been encountered.

(*c*) *Mode of production of inhibitory effects*

It had already been suggested that the inhibitory effect is independent of non-specific toxicity, and the present experiments provide further support for this view. As an example, although 2:2′-diamino-1:1′-dinaphthyl proved to be markedly toxic in experiment 94, no evidence of inhibition was detected; nevertheless 1:2′-azonaphthalene (experiments 96–98) produced considerable inhibition, with no manifestations of toxic action in the doses used. On the other hand, a few compounds (e.g. 1:1′- and 2:2′-arsenonaphthalene in experiments 160 and 162) produced a degree of inhibition which was discounted because of obvious chronic toxicity.

It is an important question whether the inhibitory property is exerted directly, or indirectly, by alterations in the anterior lobe of the pituitary gland. In a former paper (Haddow and Robinson 1937) it was concluded that 'at least a part of the general inhibition of growth produced by the carcinogenic hydrocarbons may be explicable on the basis suggested by

Zondek for oestrin, i.e. as an interference with pituitary function'. On the other hand, it seemed probable on account of the insolubility of the cancer-producing compounds that in certain circumstances their action must be restricted to the cells in the immediate vicinity. In view of the similar inhibitory effects produced on body growth, tumour growth and gonadal activity by the polycyclic carcinogenic hydrocarbons and by oestrogens carcinogenic in a restricted sense, it is likely that the mechanism in both cases is partly direct and partly mediated by the pituitary, although possibly in varying proportions. The direct inhibitory action of methylcholanthrene has been studied in vitro by Earle and Voegtlin (1938, 1940). Also, Tuchmann and Demay (1936) described complete arrest of spermatogenesis in the rat testis as a result of the direct action of 3:4-benzpyrene, although pituitary changes were also observed. For oestrogens, most attention has been paid to the functional impairment produced in the anterior pituitary (by folliculin, Zondek 1936 *b*; by stilboestrol, Noble 1938*a*). The more widespread general effect of oestrogenic compounds may in part be due to more ready absorption: Noble (1938*b*, 1939*b*) observed inhibition of body growth in rats following implantation of crystalline 9:10-dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene, triphenylethylene, 4:4′-dihydroxy-α-β-diethylstilbene, and 4:4′-dihydroxy-γ-δ-diphenyl-β-δ-hexadiene, but not following similar implantation of crystals (50 mg.) of 1:2:5:6-dibenzanthracene or 3:4-benzpyrene. Nevertheless, certain evidence shows that both natural and synthetic oestrogens can induce hormone responses locally, in the vagina or mamma, without absorption into the general circulation (cf. Robson and Adler 1940; Speert 1940; Lyons and Sako 1940). Also, Noble (1939*a*) suggested that 'whereas the action of oestrogens on the gonads seems to be indirectly produced through the inhibition of gonadotrophic hormone secretion by the pituitary gland,...body-growth may be directly affected'.

It was concluded by White and White (1938, 1939) that inhibition of the growth of the rat by certain compounds is due to a specific deficiency in the sulphur-containing amino-acids, produced by imposing on the organism an increased requirement for organic sulphur, in the form of cystine or methionine, for detoxication mechanisms. These authors produced a growth-inhibitory effect in young rats by the incorporation of 20-methylcholanthrene or 3:4-benzpyrene in the basal diet, in the proportions of 70 and 110 mg. respectively per 100 g. of ration. The dosage levels were chosen after preliminary trial had shown that such quantities would markedly retard growth for long periods without producing toxic effects. It was then shown that addition of either *l*-cystine or *dl*-methionine to the basal diet containing the methylcholanthrene or benzpyrene resulted in a prompt stimulation of the growth rate, and the same result followed subcutaneous injection of glutathione in animals ingesting the methylcholanthrene-containing diet; on the other hand, inorganic sulphur (sodium sulphate), amino-acid nitrogen (glycine), or sulphur in the form of taurine were incapable of stimulating growth under the experimental conditions. A similar relation was observed when inhibition was produced by administration of pyrene or (White 1940) of *p*-dimethylaminoazobenzene. While putting forward the above conclusion, on the basis of their own results and the data already available in the literature, White and White nevertheless emphasized the desirability of actual isolation of the mercapturic acids of these compounds, in order to place beyond doubt a relationship of growth effects to sulphur metabolism; and they also admitted the possibility that the growth-inhibitory results might not in all cases be related to a specific detoxication mechanism involving the sulphur-containing amino acids, but that some of the compounds might retard cellular metabolism.*

With regard to the mechanism of direct inhibitory action by carcinogenic compounds, some attention has also been paid to the principle discovered by Fildes (1940) and McIlwain (1940 *a*, *b*, 1941) in the case of anti-bacterial drugs. The principle relates growth-inhibitory compounds to molecular structures of nutritional importance for the organisms concerned, and thus affords a rational basis for the modelling of chemotherapeutic agents. In the present case such an application would depend on chemical relationship between the inhibitory compound and a natural vitamin or other factor essential to the growth of the animal cell: no positive experimental evidence is as yet available.

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* Paragraph added 19 August 1941.

1:4-naphthahydroquinone diacetate), Dr E. de Barry Barnett (2:3:6:7-tetramethylanthracene), Dr Ernst Bergmann (1:2:3:4-dibenzfluorene), and Dr S. H. Tucker and Dr J. Forrest (derivatives of fluoranthene, table 9).

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A study of the nuclear apparatus of bacteria

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[Plates 9–12]

Dumbbell-shaped bodies giving a positive Feulgen reaction and possessing a strong affinity for nuclear dyes have been observed in resting bacterial spores and vegetative cells from young cultures. A special study was made of the resting and germinating spores of two strains of *Bacillus mycoides*. Resting spores contain one chromatinic dumbbell body, closely attached to, but distinct from, a rod of non-chromatinic protoplasm. Division forms of this body occur in a few spores. When germination begins, the dumbbell body enters the rod of protoplasm, where it soon becomes invisible. When it reappears at a later stage of the germination process, it has divided into two closely contiguous dumbbell bodies. Further divisions of the chromatinic bodies precede the divisions of the vegetative cells in a regular way. Each vegetative cell usually contains a pair of dumbbell bodies. Similar chromatinic bodies have been demonstrated in the cells of *Proteus vulgaris* and two Sarcinae. The two single Feulgen-positive bodies described by previous authors in the resting cells of various spore-forming and non-sporing organisms thus appear to consist of two pairs of dumbbell bodies, separated by a delicate boundary. The single 'cells' to which these writers refer are in fact short, two-celled filaments. It is concluded that the dumbbell bodies are comparable to the chromosomes of plant and animal cells.

INTRODUCTION

> The great majority of bacteria which have been described have taught us nothing concerning the internal structure of the bacterial cell. Nearly all pathogenic forms are of exceedingly small size; and in addition to this great disadvantage they have mainly been studied after fixing and staining in the usual bacteriological manner, which renders them worthless for cytological purposes. It is desirable in the first place to study the largest and most easily investigated forms and to examine them after treatment by suitable cytological methods.
>
> C. C. DOBELL, *Quart. J. Micr. Sci.* **56** (1911), 397.

Since these lines were written important advances have been made in our knowledge of the nuclear apparatus of bacteria. These advances have been mainly due to the application to bacterial cytology of the Feulgen process for the detection of thymonucleic acid (Feulgen 1926). For details of this method and a critical discussion of its advantages and limitations the reader is referred to an article by H. Bauer (1932) and to a recent paper by Hillary (1939). A full bibliography has been published by Milovidov (1938).

Since considerable quantities of nucleoprotein have been extracted from many different bacteria, it is hardly surprising that bacteria should give a positive Feulgen reaction. Positive results consisting of a diffuse, faint mauve coloration throughout the bacteria were obtained by Voit (1925, 1927) on various spore-forming and non-spore-forming bacteria, by Pietschmann and Rippel (1931, 1932) on *Bacillus mycoides* and by Stapp and Bortels (1931) on *Pseudomonas tumefaciens*.

Recently, however, many cases have been reported in which the Feulgen reaction was restricted to discrete bodies of regular size and shape. Such structures have been found in *Bacillus anthracis* (da Cunha and Muniz 1929), *Vibrio cholerae* and *Bacillus paratyphosum* B (Korobkova 1931–4), *Pasteurella pestis* (Pokrowskaja 1930–31), *Bacterium coli* (Kuhn and Sternberg 1931), some Sarcinae (Petter 1933; Milovidov 1935; Sassuchin 1935; Stille 1937; Piekarski 1937), nine different cocci (Delaporte 1936) and *Mycobacterium tuberculosis* (Epstein, Ravich-Birger and Svinkina 1936).

The most important observations, however, are those of Stille (1937) on *Bacillus subtilis* and other aerobic spore-forming bacilli and of Piekarski (1937–9) on *Bacterium coli*, *Bact. typhosum*, *Bact. paratyphosum* B, *Proteus vulgaris* and *Bacterium prodigiosum*.

According to both Stille and Piekarski, Feulgen-positive bodies of regular size and shape lie at either end of the bacterial cell and grow and divide with the organism, this division of the bodies preceding that of the cell. According to Piekarski, *Bact. paratyphosum* B and other non-sporing bacteria occur in culture in two forms. One form, with two Feulgen-positive bodies, is obtained in cultures of 3–6 hr. incubation, and the other, which is smaller, with only one Feulgen-positive body, is found from 6 hr. onwards. In subculture the 'binucleate' form is somehow derived from the smaller 'uninucleate' form.

Feulgen-positive structures have also been demonstrated in bacterial spores. It is usually stated that these structures are not inside the spore, but are attached to its outer surface. Pietschmann and Rippel (1932) have described single granules, dumbbell-shaped bodies (probably representing two closely adjacent granules), and small curved rods, adhering to the spores of *Bacillus mycoides*. Milovidov (1935) obtained similar results with spores of *B. mycoides*, *B. megatherium* and *B. anthracoides*, and Schaede (1939) with *B. mycoides* and *B. megatherium* spores. On the other hand, Stille (1937) saw a single peripheral granule attached to the *inner* surface of the spore membrane in an organism 'belonging to the megatherium group'. Three months later the granule was seen in the centre of 88 % of the spores. Stille, who believes that the vegetative cells possess two Feulgen-positive bodies, states that one of them is included in the spore, while the other perishes with the spore mother cell.

Interesting observations on nuclear structure in bacteria have also been made by means of non-specific staining methods. Nakanishi (1901) described structures which he believed to be nuclear in *Bacterium coli*, *B. anthracis*, *Bacillus subtilis* and many other organisms. Their size, mode of division and distribution in the cell correspond closely with those subsequently demonstrated by the Feulgen technique in the same organisms by Stille and Piekarski. Stoughton (1929, 1932) used carbolfuchsin as fixative and stain and describes an 'internal central structure which passes through a divisional cycle which is correlated with the division of the cell body and which is suggestive of nuclear division'. It is probable that the structures observed by Stoughton are identical with the Feulgen bodies.

Badian (1933), working with Giemsa's stain and rapid differentiation in 1 % eosin, discovered regularly arranged groups of chromatinic bodies, which he regards as chromosomes, in the cells of *B. mycoides* and *B. subtilis*. These chromosomes, which are dumbbell-shaped, occur singly, or in double pairs according to the various stages of division, and one chromosome is said to take part in the formation of the spore, though it is not visible in the finished product. Badian's photomicrographs are not clear enough to be convincing and his findings do not seem to have been reinvestigated, but they link up clearly with those of Piekarski (1937), who was able to demonstrate his 'nucleoids' in *Bacterium paratyphosum* B by Badian's method, though they appeared larger and less well defined than in Feulgen preparations.

Allen, Appleby and Wolf (1939), who used a vital staining method, have described certain configurations, in the young vegetative cells and germinating spores of an organism isolated from grass, which they believe to represent a meiotic process.

In the present investigation the cytology of various bacteria and the changes which their internal structure undergoes during growth have been studied in detail. Special attention has been paid to the resting and germinating spores, the cytological structure of which has not hitherto been adequately described. These results were obtained largely by means of Piekarski's modification of Giemsa's staining method. Although this author's own observations seem to be based almost entirely on Feulgen preparations, he mentions that in the cells of various non-sporing organisms treated with 1/N HCl at 60° C,* 'nucleoids' can be demonstrated with Giemsa's stain, although such cells appear homogeneous if stained with Giemsa's solution without this preliminary treatment in acid. In the present study Piekarski's modification of the Giemsa method has proved more valuable than the Feulgen technique. Constant comparison of parallel preparations has shown a close correspondence between the structures stained by the two methods, but the Giemsa preparations are optically superior and, in particular, were found to reveal the internal structure of spores with beautiful distinctness, so that several new cytological features of the germinative process were disclosed.

MATERIALS AND METHODS

Materials

The following organisms were investigated cytologically:

Bacillus mycoides	A typical strain, No. 16, was obtained from the National Collection of Type Cultures (N.C.T.C.). A variant, No. 1110, was kindly supplied by Dr den Dooren de Jong, Rotterdam, who isolated it from soil.†

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* For the sake of convenience, if somewhat inexactly, this treatment will be referred to as 'hydrolysis'.

† This organism is one of the bacteriophage-producing strains of spore-forming bacilli described by den Dooren de Jong, who classified it (1933, 1934) as a strain of *Bacillus mycoides* because of the lytic action of its 'phage on several typical *mycoides* strains. It is now kept in the N.C.T.C.

B. megatherium	No. 5635, N.C.T.C. and No. 899, den Dooren de Jong.
B. subtilis	Nos. 2586 and 3610 (Marburg), N.C.T.C.
B. mesentericus	No. 4, N.C.T.C. and No. 86 from the Botany School, Cambridge.*
An aerobic spore-forming organism isolated from grass	Described by Allen, Appleby and Wolf (1939) and kindly supplied by Dr Appleby.
Spores of Bacillus 'Soil 2'	A large, spore-forming, aerobic bacillus, isolated and kindly supplied by Dr J. W. Howie, who used it in experiments on the antigenic properties of spores (Howie and Cruikshank 1940).
Proteus vulgaris	No. 18 from the Botany School, Cambridge, and No. 5821 from the N.C.T.C.
Sarcina lutea	From a nutrient agar plate which had been exposed to the air.
Sarcina aurantiaca	No. 01346/952, N.C.T.C.
Yeasts: *Torulopsis bacillaris*†	Obtained from the 'Bureau voor Schimmelcultures', Delft, Holland.
Monilia albicans	A strain was kindly provided by Professor L. P. Garrod, St Bartholomew's Hospital, London.

Methods

Resting spores.

Pure spore cultures were obtained on potato agar at 37° C. The period of incubation required for different organisms varied from a few days to several weeks. All microscopic preparations were made on cover-slips. Fixation in osmic vapour was used almost exclusively. A concentrated atmosphere of osmic vapour was obtained by placing 4–5 ml. of 2 % osmium tetroxide in a covered dish about 1 in. deep containing several layers of glass balls. The cover-slips bearing the moist films were laid face upwards on the glass balls, above the level of the solution, for periods varying according to the proposed subsequent treatment, dried in air, and transferred to 70 % alcohol, in which they could be kept indefinitely. Resting spores can also be satisfactorily fixed in Schaudinn's sublimate alcohol, or Bouin's fluid. The films were then hydrolysed in N/1 HCl at 60° C for 7 min. Subsequent treatment was by one of the following methods:

(*a*) *Schiff's reagent*, according to the usual Feulgen technique (see *The Microtomist's Vademecum*, 10th ed. 1937). For this purpose films fixed for 20 min. in osmic vapour should be used. The light brown tinge which osmic vapour gives to the cytoplasm often makes counterstaining unnecessary, but it was usually found helpful to counterstain for about

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* The author is indebted to Dr W. J. Dowson for various strains from his collection at the Botany School, Cambridge.

† A strain is now kept in the N.C.T.C.

30 sec. in a mixture of 0·5 % light green and 0·5 % naphthol green in 90 % alcohol. The films were dehydrated in 70 %, 96 % and absolute alcohol, cleared in xylol and mounted in canada balsam.

(*b*) *Giemsa's stain.* Films fixed in osmic vapour for 3–5 min. and hydrolysed as above were floated on dilute buffer solution (Soerensen's M/200) of pH 6·9–7·0 (Balint 1926) containing one drop per ml. of Gurr's improved Giemsa's stain R 66 for 1 hr. at room temperature, or ½ hr. at 37° C. The depth of the stain was judged by examining the film in buffer solution with a water immersion lens. The preparation was slightly overstained and differentiated in the following series of mixtures of acetone and xylol: 20:1 (2–4 sec.), 14:6 (10 sec.) and 6:14 (10 sec.), and examined in xylol with a 1/12th oil immersion lens. Differentiation was continued until the chromatinic bodies (vide infra) were distinct, when the film was mounted in Gurr's neutral canada balsam.

(*c*) *Iron haematoxylin.* Films fixed as for method (*b*), or with sublimate alcohol, were mordanted with 2·5 % iron alum for 2 hr. at 37° C, or overnight at room temperature, and after being rinsed in distilled water were stained with Heidenhain's haematoxylin for the same period. The preparations were again rinsed and examined in distilled water with a water immersion lens. Slight differentiation in a weak solution of iron alum was sometimes necessary; some of the films were left rather darkly stained to show the spore membrane and others differentiated to give the sharpest possible image of the chromatinic bodies. The preparations were washed in running tap water for 20 min., dehydrated, cleared and mounted as under (*a*). Iron haematoxylin exaggerates the size of the chromatinic bodies and tends to blur their outlines, but gives a sharp contrast between cytoplasm and chromatinic matter.

Germinating spores.

For the early stages of germination (1½–10 min. incubation) the following method was used. Broth, a pipette and the cover-slip bearing a dried film of resting spores were warmed in the incubator for 15 min., after which two or three drops of broth from the pipette were spread over the whole spore film. Cultures were removed from the incubator at intervals of 1½, 2, 3 and 5 min. For later stages (10 min. to 1 hr. incubation) spore films were partly covered with nutrient broth and the cover-slips mounted as hanging drops over hollow-ground slides. After incubation most of the broth was shaken off and the film fixed in osmic vapour (8 min. for Feulgen, 3–5 min. for Giemsa) and 70 % alcohol, hydrolysed as under (*a*) or (*b*) above. Iron haematoxylin was found to give poor results for germinating spores.

Vegetative cells.

Once the spores have germinated the young vegetative cells tend to detach themselves from the cover-slip, so that in hanging drop preparations it is rare to find any two cells at the same focal level. Better results were obtained from impression preparations, or by a modification of E. Klieneberger's agar fixation method (1934). Impression preparations were made on cover-slips from nutrient agar cultures, fixed in osmic vapour (8 min. for Feulgen, 3–5 min. for Giemsa) and 70 % alcohol, hydrolysed and treated as under (*a*) or (*b*) above. Special care should be taken to avoid overstaining vegetative cells as decolorization is laborious and unsatisfactory.

The agar fixation method gave less crowded preparations than impression films. Three or four narrow strips of agar were gently placed on a dried film of resting spores. The film was incubated for 1½–2 hr. at 37° C in a moist chamber. After incubation the film was fixed in osmic vapour as described above and 70 % alcohol. The agar strips either floated off in the alcohol, or were easily detached with a needle.

Fixation with Bouin's fluid, though unsuitable for the study of the chromatinic bodies of vegetative cells, was excellent for the demonstration of cell boundaries (vide infra). For the latter purpose the cells were fixed for 20 min. in Bouin's fluid, rinsed in 70 % alcohol and stained for 3 min. with Giemsa's solution or with iron haematoxylin *without* previous hydrolysis.

RESULTS

Bacillus mycoides

Resting spores.

An account of the cytological changes in the bacterial life cycle may conveniently begin with a description of the structure of the resting spore, for the study of which two strains of *B. mycoides* were found particularly favourable.

None of the ordinary staining methods reveal the internal structure of spores (figures 1 and 16, plate 9), but treatment with warm hydrochloric acid before staining shows that they consist of two parts: an apparently structureless rod of protoplasm and a small, usually dumbbell-shaped, body which is closely attached to, but distinct from, the rod (*B. mycoides* 16, figures 7, 8, 11, plate 9; *B. mycoides* 1110, figures 2–6, 9–11, plate 9, figure 46, plate 12, and figure 52 A. The position of the dumbbell-shaped body relative to the rod varies slightly in different spores. These two constituents of the spore stain quite differently from each other. After hydrolysis the rod gives a negative Feulgen reaction, stains faintly with iron haematoxylin and clear blue with Giemsa's solution. On the other hand, the dumbbell-shaped body gives a positive Feulgen reaction (figure 10, plate 9), stains black with iron alum haematoxylin and red or purple with Giemsa's solution. The spore membrane, which is almost invisible in hydrolysed preparations, will be considered later.

The chromatinic* body is probably always dumbbell-shaped, and apparent divergences from this form may usually be attributed to variations in the position in which the spore is lying. Pairs of closely contiguous dumbbell bodies, suggestive of division, as well as rings and crescents of chromatinic material, were found in a few resting spores of *B. mycoides* 16, the typical strain (table 1), but were more frequent in the spores of *B. mycoides* 1110, the variant strain.

The internal structure of the spores of *B. mycoides* varies with the age of the culture in a manner which is still under investigation. The description given in the preceding paragraphs applies to spores kept for several months at room temperature. It is hoped that a study of spore formation will supplement the present incomplete picture of the organization of the spore, explain how the chromatinic dumbbell body reaches its position outside the spore's cytoplasmic core, so clearly visible in the case of *B. mesentericus* (figures 12, 13, plate 9), and the relation of the two spore membranes (vide infra) to the two internal structures.

Germinating spores.

(*a*) 0–5 *min. incubation.* When the spores are incubated in a drop of broth at 37° C, during the first few minutes they lose their refractility, enlarge slightly and with ordinary staining methods acquire a strong affinity for dyes (figure 16, plate 9). After 30 min. incubation they begin to elongate, and after about an hour they burst their spore cases and are gradually transformed into the familiar vegetative filaments with rounded ends and glassy, homogeneous appearance, which when stained by the usual techniques betray no trace of structural differentiation. In hydrolysed Giemsa-stained preparations, however, the following internal changes can be followed during germination.

During the first 5 min. of incubation the apparent division forms (rings, parallel dumbbell-shaped bodies, V-forms and crescents) which, as mentioned above, occur in a certain proportion of the resting spores, greatly increase in number (table 1). Many dumbbell bodies are now somewhat larger than in resting spores and more than ever distinct from the nonchromatinic rod. Soon, however, most of them become transformed into what appear to be chromatinic rings. The ring form, which is rare in the resting spores, is by far the commonest structure in the germinating spores. The transformed chromatinic bodies then leave their peripheral position and *enter* the rod of non-chromatinic cytoplasm, meanwhile changing their colour from bright red to purple. Those chromatinic bodies which are not already in the ring form when they enter the cytoplasm immediately assume this configuration after entry (figure 52B; figure 21, plate 9; figure 22, plate 10).* Within the spore protoplasm the chromatinic rings

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* This term will be used because of the strong affinity of the structure for nuclear stains after hydrolysis.

TABLE 1. CHANGES IN THE STRUCTURE OF SPORES OF *B. MYCOIDES* DURING 5 MIN. INCUBATION WITH NUTRIENT BROTH AT 37° C

		total number of spores counted	number of spores with *dividing* chromatinic bodies (rings, crescents and V-forms)	%
resting spores	film 1	516	25	5
	film 2	514	21	4
after 1½ min. incubation	film 3	622	116	18·5
	film 4	568	108	19

After 5 min. incubation most of the spores are devoid of visible structural differentiation and stain uniformly light blue.

		total number of spores counted	number of spores stained uniformly light blue	%
after 5 min. incubation	film 5	665	546	82
	film 6	571	410	72

A few spores still stain differentially and in these most of the chromatinic bodies are now in a stage of division.

		total number of differentially stained spores counted	number of spores with dividing chromatinic bodies (rings, crescents and V-forms)	%
after 5 min. incubation	film 5	414	382	93
	film 6	400	272	68

Two films were counted in each group. The figures of the last two groups were obtained from the same pair of films.

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* In the cytoplasm of many spores incubated for a few minutes, a central body is distinguishable which stains more darkly than the surrounding protoplasm (figure 17, plate 9). It could not be identified in Feulgen preparations and its significance is at present unknown.

widen until they nearly coincide with the contours of the spores and gradually become invisible. At this stage the spores appear as boat-shaped cells, stained uniformly light blue and lacking visible structural differentiation (compare figures 7, 8, 11, plate 9 with figure 23, plate 10 and

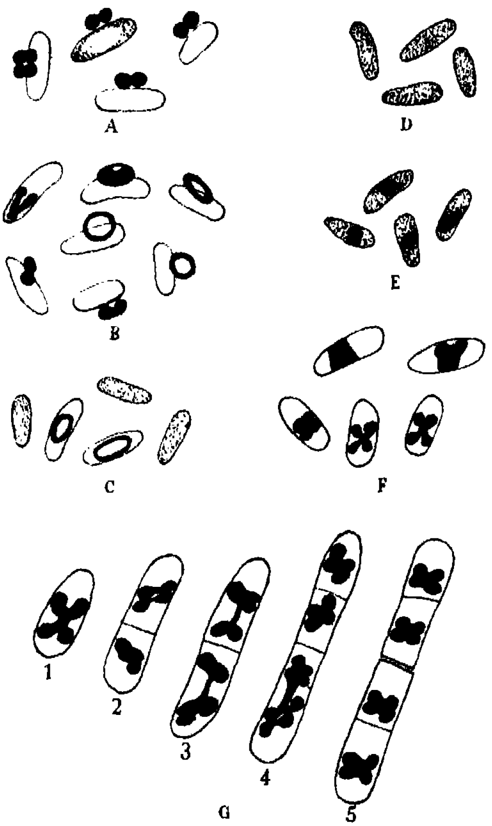

FIGURE 52. Diagrams of the transformation of resting spores of *Bacillus mycoides* into vegetative cells as seen in preparations fixed in osmic vapour, treated with N/1 HCl and stained with Giemsa's solution. Surrounding spore membranes invisible. Minutes give time of incubation at 37° C. Figures in brackets refer to the photomicrographs on plates 9 and 10 which the diagrams attempt to explain. (A) Resting spores. (B) 1½ min. Ring formation and entry of rings into the non-chromatinic rods (cf. figures 17, 19, 21, plate 9). (C) 2½ min. Widening of the rings inside the cytoplasm (cf. figure 22, plate 10). (D) 5 min. All spores uniformly stained (cf. figure 23, plate 10). (E) 20 min. Central chromatinic structures have reappeared (cf. figure 24, plate 10). (F) 40–60 min. Different types of chromatinic structures in young vegetative cells (cf. figures 25–28, plate 10). (G) 75 min. and later. Successive division stages of the chromatinic structures and cell divisions leading to the formation of two two-celled filaments (cf. figures 30–35, plate 10). 1 (cf. figure 30, cell on the left), 2 (cf. figure 30, cell on the right), 3 (cf. figures 31–33), 4 (cf. figure 33), 5 (cf. figures 34 and 35).

figure 52D). Table 1 illustrates these changes. Chromatinic bodies are visible in all resting spores of *B. mycoides*. In 4–5 % of spores these bodies already show signs of division, and after 1½ min. incubation this percentage rises to 18–19 %. At 5 min. the chromatinic bodies have disappeared from most of the spores, leaving them with a faint, diffuse staining reaction, while in the few spores in which chromatinic bodies are still visible they are nearly all dividing.

Incubation of *mycoides* spores in distilled water for 3 hr. resulted in no visible changes in the spores' internal organization, neither is the morphology of dried spores altered by incubation, even for several hours. This is evident from the fact that spores in films dried for 20 min. in the incubator do not differ from spores dried at room temperature, also from the normal appearance of resting spores in preparations of vegetative cells grown at 37° C by the agar strip method, where areas of dried spores alternate with areas covered by nutrient agar. The 15 min. preliminary warming of the spore-bearing cover-slip, described in the section on technique, can therefore be disregarded in the description of the changes characterizing the first few minutes of incubation with nutrient broth. Cultivated in broth at room temperature the spores required 3–4 hr. or more to germinate. The slowness of the whole process under these conditions may well prove an advantage for the cytological study of early germination changes. No such experiments, however, have yet been attempted.

Feulgen preparations faithfully reflect the appearances seen in those stained with Giemsa's solution, the chromatinic bodies being definitely Feulgen positive. The strength of the reaction increases during the first 1½ min. incubation, then diminishes, and after 5 min. incubation most of the spores give a diffuse, faintly positive Feulgen reaction throughout.

If a film of spores is partly covered by a drop of nutrient broth the spores outside the area covered by the fluid remain unchanged, but those nearest to the extreme margin of the drop start germinating, though they never complete the process even after 1½ hr. incubation. Frequent among these cases of arrested germination are spores like those photographed in figure 20, plate 9, with the peripheral dumbbell body halfway inside the non-chromatinic rod. It may be that the divided dumbbell bodies and ring forms which, as described in the preceding section, occur in a small number of resting spores, are also stages in a premature and frustrated germination process.

(*b*) 5–75 *min. incubation*. The normal, uniformly stained spores seen after 5 min. incubation (figure 23, plate 10; figure 52D) remain in that condition for about 15 min. Then specks of chromatinic material begin to appear in the centre of the cells (figure 24, plate 10) and gradually increase in size and distinctness until after 40 min. incubation they have formed broad bands (figure 25, plate 10 and figure 52F). By the end of the first hour these chromatinic figures give a very intense Feulgen reaction and, in Giemsa preparations, stand out as deep, brilliant purple structures contrasting sharply with the almost colourless 'cytoplasm' of the now greatly enlarged, rod-shaped spores (figures 26, 27, 33, plate 10).

The chromatinic structures at this stage consist of two dumbbell bodies arranged in one of the three following ways:

(1) A 'tetrad' figure of marked symmetry in which the four round bodies of the two dumbbells have the same size and form (figure 28, plate 10 and figure 52 F).

(2) *X*-figures formed by two curved dumbbell-shaped bodies. It is not always possible to decide their orientation with reference to the long axis of the cell (figure 52 F).

(3) Broad bands of chromatinic matter composed of a pair of dumbbell bodies diverging slightly towards one side of the cell; this is the commonest type (figure 52 F and figures 25, 27, plate 10).

There is no hard and fast distinction between these three types, which merge into one another through a series of intermediate forms.

Vegetative cells.

(*a*) *First 7 hr. incubation.* During the first 15 min. of the second hour of incubation the newly hatched* vegetative cells elongate and the chromatinic structures within them divide (figure 52 G and figures 29, 30, plate 10). The plane of division is usually at right angles to the long axis of the cell and the two chromatinic structures move to opposite ends of the organism, leaving strands of chromatinic matter stretched out between them.

Some of the *X*-figures seem to divide rather differently. Their long axis appears to be orientated parallel to the *long* axis of the cell, and as the cell grows the chromatinic bodies become stretched into thin threads with clubbed ends. At this stage a crossing-over occurs, followed by breaking of the threads at the point of crossing. Two V-shaped bodies result which presumably contract and straighten out to form short dumbbells orientated parallel to the *short* axis of the cell, since only this type of chromatinic figure is found in the succeeding generations of vegetative cells.

The cytological analysis of these figures is not yet complete, but they are too frequent among the cells of the first vegetative generation not to be mentioned here. If my interpretation of the *X*-figure is correct, then the crossing-over serves merely to align the plane of division of the chromatinic figure with that of the cell; there is neither need nor evidence for regarding it as part of a meiotic process.

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* Iron alum haematoxylin preparations show that the spore membrane bursts and is discarded at this stage. It is, however, invisible in hydrolysed, Giemsa stained preparations.

The first division of the young vegetative cell results in the formation of a short rod composed of two cells separated by a delicate boundary stained a faint pink (figure 52 G (2), figure 30, plate 10). Each cell contains a fairly easily resolved pair of dumbbell-shaped chromatinic bodies which give a distinct but rather weaker Feulgen reaction than the single chromatinic body in newly hatched single vegetative cells.

The two-celled rod elongates, the chromatinic structures in each component divide (figures 31–33, 36, plate 10) and cell boundaries appear between the products of division. At this stage, when growth takes place on a solid medium, the single rod usually breaks into a pair of two-celled daughter rods (figure 1 G and figures 34, 35, plate 10), so that four-cell filaments (figure 37, plate 10) are rare. Occasionally, however, breakage occurs at a stage corresponding to figure 52 G (4) and this produces cells with incompletely divided figures (figure 35, plate 10, asterisk). This subdivision of the filaments or rods of aerobic spore-forming bacteria into two or four cells has previously been described for *Bacillus megatherium* (de Bary 1884), *B. anthracis* (Nakanishi 1901), *B. subtilis* (Kynasi 1930) and *B. mycoides* (Badian 1933). In certain species it becomes particularly obvious during spore formation (figure 45, plate 12).

The relation between the division of the chromatinic structure and that of the cell during the first few hours of incubation may be summarized as follows. The resting spore possesses a single dumbbell-shaped chromatinic body. This body divides longitudinally during spore germination without a corresponding division of the cell. Thereafter each cell division is preceded by a division of each of the two components of the paired chromatinic structure.

(*b*) 7–24 *hr. incubation.* The well-defined chromatinic figures which are always found in the vegetative cells during the first few hours after germination are absent in the cells of cultures which have been incubated on nutrient agar slants for 24 hr., or kept at room temperature for several days. After 7–10 hr. incubation at 37° C, cells with irregularly dispersed chromatinic material are abundant and at still later stages the culture consists chiefly of long filaments some of which contain a few very small, deeply staining chromatinic granules which give a weak positive Feulgen reaction. Except for these not very obvious granules the cells are uniformly stained. This accounts for the fact that so many workers have failed to observe any internal structure in bacteria cultivated overnight. The cause and significance of the almost complete disappearance of chromatinic material from the vegetative cells after such a relatively short incubation period is not yet understood. Further study of these later changes and of the behaviour of the chromatinic bodies during spore formation is in progress.

There is one type of cell which is common among early vegetative cells, but rare among germinating spores or in cultures incubated for more than a day. It is as long as 2–4 normal cells and is characterized by a central clump of chromatinic material. The origin of these cells is somewhat obscure. Badian, who also observed them in *B. mycoides*, believes they are the products of autogamous fusion of the chromosomes of the normal cells, and I have observed many cells to which this interpretation might well apply. In my own preparations apparent divisions of the central clump of chromatinic material have been seen, and also cells connected by thin strands of chromatin with the central masses of adjacent cells, the appearance suggesting a nearly completed division. In some the chromatinic material is obviously pycnotic while in others it has a frayed edge and dust-like particles are distributed throughout the protoplasm. These appearances suggest that at least some of these abnormal cells are disintegrating. The fate of others is uncertain.

A closely similar type of cell is a regular stage in the rejuvenation of old cultures. If cells from an old culture are transferred to fresh medium differentiation into Feulgen-positive chromatinic material and Feulgen-negative cytoplasm is re-established in some of the filaments after a lag of 2–3 hr. A deeply staining, dense clump of chromatinic substance first appears which, in the course of several cell divisions, gradually breaks up into smaller units corresponding in size and distribution to the chromatinic structures which characterize the first few hours of vegetative growth following spore-germination. This curious process of rejuvenation will be considered in greater detail in a subsequent communication.

It is interesting to note that under certain conditions the usual uniform staining of cells from young vegetative cultures can also be obtained *after hydrolysis*. If hydrolysed vegetative cells are mordanted with iron alum and stained with Heidenhain's haematoxylin they appear an even bluish grey, although this method demonstrates the chromatinic body in spores. The same homogeneous appearance can be produced with Giemsa's solution, if hydrolysis is followed by treatment with a watery solution of tannic acid (figure 47, plate 12).

The type of chromatinic structure found in *B. mycoides* occurs very widely among bacteria, as will be seen from the following notes on other spore-forming organisms, *Proteus vulgaris* and some Sarcinae.

Bacillus mycoides 1110

The spores of this bacillus are on the average considerably larger than those of the typical strain, but have essentially the same internal structure and germinate in a similar way. The chromatinic structures of the vegetative cells are less well defined than those of *B. mycoides* 16 (figures 38, 39, plate 11).

Bacillus megatherium

The spore membrane is very difficult to distinguish in *B. mycoides*, though sometimes visible in strongly stained, hydrolysed preparations examined in water, but in *B. megatherium* the membrane stains intensely with Giemsa's solution, and laborious decolorization is required before the structures of the interior are clearly visible. In addition a delicate, ill-fitting envelope encloses the whole spore* and still further obscures the internal structure. These disadvantages do not apply to the staining of hydrolysed spores with iron haematoxylin; but on the other hand this stain tends to exaggerate the size of the chromatinic bodies, which are actually much smaller than those of *B. mycoides*, both absolutely and in relation to the size of the cell. Giemsa preparations of the hydrolysed vegetative cells are also difficult to differentiate satisfactorily. The Feulgen technique gives the best results with this organism and leaves no doubt that its internal chromatinic structures are essentially the same as in other aerobic spore formers.

Bacillus 'Soil 2' (Howie and Cruickshank)

The chromatinic bodies in the resting spores of this organism will not stain with Giemsa's solution even after hydrolysis, but are clearly seen in preparations stained with Feulgen's method or iron haematoxylin. They closely resemble those of *B. megatherium*.

A peculiarity of *Soil* 2 germinating spores is the early detachment of the spore membrane, sometimes before the chromatinic body has entered the cytoplasm, this proving definitely that the body is enveloped by and not superficially attached to the spore membrane (figure 15, plate 9).†

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* Such an envelope has been described by Nakanishi (1901) for *Bacillus anthracis* and by Allen, Appleby and Wolf (1939) for another aerobic organism. It can also be demonstrated with iron haematoxylin around the spores of *B. mycoides*. Presumably it represents the shrivelled remains of the spore mother cell.

† v. Klinckowstroem (1931), using carbolfuchsin and gentian violet, has described, in the deplorable terminology of Enderlein (1925), two 'mych' beneath the capsule of the germinating spores of a large aerobic bacillus related to *B. subtilis*. His figures 86–88 suggest that the 'mych' are identical with the chromatinic bodies of the present investigation.

Bacillus mesentericus

The resting spores contain a single, round, deeply stained chromatinic body, well separated from the lightly stained cytoplasm in Giemsa preparations (figures 12, 13, plate 9). Observations on the germinating spores and vegetative cells of this organism make it probable, however, that the appearance of a single chromatinic body is due to faulty resolution of a pair of minute, closely contiguous granules. Regarding the distribution of chromatinic bodies in them, the vegetative filaments closely resemble the somewhat broader cells of *B. mycoides*. In impression preparations in which the cylindrical rods have probably become flattened while drying on the glass surface, the apparently homogeneous chromatinic bodies can be resolved into pairs of dumbbell bodies like those of *B. mycoides* and related species. No cell boundaries separating the chromatinic bodies are visible in hydrolysed Giemsa-stained preparations. The cell boundaries, however, can be clearly demonstrated by Bouin fixation and staining with iron haematoxylin and in such preparations the vegetative filaments are seen to be subdivided into as many cells as there are resting or dividing chromatinic bodies. Thus the two filaments marked 'a' in figure 41 consist each of two cells and correspond to figures 31, 33 of *B. mycoides*.

Bacillus subtilis

Spores and vegetative cells contain essentially the same chromatinic structure as those of *B. mesentericus*.

Proteus vulgaris

The occurrence of dumbbell-shaped chromatinic structures is not confined to the spore-forming bacteria. In preparations of *Proteus vulgaris* obtained by the agar fixation method from cultures incubated for 2–6 hr., hydrolysed and stained with Giemsa's solution, the slender rods are seen to contain round chromatinic bodies (figures 43, plate 11), closely resembling those demonstrated by the same stain in *Bacillus mesentericus*. These observations confirm those of Piekarski (1939) on *Proteus* and other non-sporing organisms. In impression preparations, however, as in the case of *Bacillus mesentericus*, the 'nucleoids' can be resolved into the same pairs of dumbbell bodies already described in the spore-formers (figure 44*a*, *b*, plate 11).

Cell boundaries between the chromatinic structures can be demonstrated in *Proteus vulgaris* after Schaudinn or Bouin fixation followed by a few minutes staining with Giemsa's solution. From such preparations it is evident that the short rods (stage 1 in figure 44*a*, plate 11) are composed of two cells, each containing a pair of chromatinic bodies.

Sarcinae

Clear cytological results were obtained with a yellow *Sarcina* isolated from an agar plate exposed to the air and with a strain of *S. aurantiaca* from the National Collection of Type Cultures.

Central Feulgen-positive granules have often been demonstrated in Sarcinae (Sassuchin, Milovidov, Pette, Piekarski), but the number of granules present in each cell was uncertain. To investigate this point in the Sarcinae mentioned above the following method was employed.

Smears of the organism from agar slopes were fixed in osmic vapour and allowed to dry. The cover-slip bearing the dried cells was mounted in distilled water on a thin slide and sealed with paraffin wax. A suitable group of cells with all its elements in nearly the same focal plane was selected and photographed. The cover-slip was then removed and the preparation was stained by the Feulgen method, dehydrated, mounted in Canada balsam and the selected group of cells was again photographed. The first photograph showed clearly the outline of the cells, which is difficult to determine in Feulgen preparations, and the second photograph the distribution of the chromatinic bodies. A comparison of the two pictures (figures 48*a*, 48*b*, 49*a*, 49*b*, 49*c*, plate 12) established the fact that many cells contain two chromatinic bodies. This observation was confirmed by examining water-mounted smears by dark-ground illumination, when two perfectly round, dark points were distinguishable in every cell which presented itself at a favourable angle.

It seems certain, therefore, that in the Sarcinae I have studied each cell normally contains two chromatinic bodies. Presumably the two granules are connected by a strand of Feulgen-negative material since they are always the same distance apart. Division of the chromatinic material seems to be by longitudinal fission.

The structure of the Feulgen-positive bodies described above corresponds closely with that of the nuclear structures described in various Sarcinae by Dobell (1911) and Meyer (1912). Whether these bodies are comparable to the chromatinic structures in the spores and young vegetative cells of the aerobic spore-forming bacteria, though very probable, is not yet certain.

Bacillus malvacearum

Reinvestigation of Stoughton's observations on *B. malvacearum* (1929, 1932) with the methods employed in the present study has not been very successful. Feulgen-positive structures were regularly found in cells from cultures several weeks old, but it is not yet clear what relationship these bear to the structural elements demonstrated by that author.*

Yeasts

The differentiating effect of hydrochloric acid is not confined to bacteria. The nuclei of four different *yeasts*, two of which are illustrated in figures 50 and 51, plate 4, stained much more clearly with Giemsa's solution and contrasted much more sharply with the cytoplasm if the cells were hydrolysed before staining. As is well known, the nuclei of yeast cells give a positive Feulgen reaction (Rochlin 1933; Imseneckij 1936). After fixation with osmic vapour, 7 min. hydrolysis, the time found to be optimal for bacteria, is also satisfactory for yeast nuclei. This parallelism in the general staining properties of the chromatinic structures of bacteria and those of the recognized nuclei of yeast cells provides evidence in favour of the nuclear nature of the former.

The thinness of the stalk connecting the buds with their mother cells in figures 50, 51, plate 12, is an apparent one and due to the fact that the strong membrane surrounding the cell is too faintly stained to be visible. It is, however, quite distinct before and after hydrolysis when fixed yeast cells are examined in water.

DISCUSSION

The literature on nuclear structures in bacteria before 1911 and 1912 has been critically reviewed by Dobell and A. Meyer respectively. More recently the subject has again been surveyed in detail by Pietschmann and Rippel (1931). A brief discussion of some of the older work will therefore be sufficient here.

The claim to nuclear character for the small granules which A. Meyer (1899) demonstrated in the vegetative cells of *Bacillus tumescens* with his Formol-Fuchsin treatment, and to which reference has frequently been made in the past, can now be dismissed. The Feulgen-positive chromatinic structures which by analogy with other large spore-formers can safely be assumed to have existed in Meyer's bacterium, would have appeared much larger and possessed an entirely different shape. Looking back on Meyer's (1893, 1899, 1908, 1912) much-quoted work, one cannot help wondering how the granules which he demonstrated by means of quite unspecific staining methods, without ever providing evidence of their ability to divide, could have been considered to represent nuclear structures. The small chromatinic body which proper cytological methods allowed him to stain in the centre of the spore rudiment of '*B. pasteurianum*' has to be taken more seriously, and will be discussed in a proposed account of the behaviour of the chromatinic dumbbell bodies during spore formation.

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* Since this was written, Dr W. J. Dowson of the Botany School, Cambridge, has been able to demonstrate typical paired chromatinic dumbbell bodies in the cells of an 18 hr. culture of *B. malvacearum*.

Dumbbell structures accredited with nuclear character have been observed on various occasions in the past. Swellengrebel's (1907) account of the division stages of such structures in '*B. binucleatum*' leaves no doubt that he was looking at equivalents of the chromatinic dumbbell bodies described in this communication.

Among Mencl's (1906) drawings of the internal structure of various bacteria from the Prague watermains are many examples of the type of nuclear structure described in the present investigation. Unfortunately this author failed to distinguish between these and the many large 'volutin' granules which were present in his material.

Recently the introduction of the Feulgen method into bacterial cytology has greatly clarified the situation and has shown that in many bacteria the nuclear apparatus conforms to a simple, ever-recurring pattern. Use of the Feulgen method only, however, has sometimes given misleading results and in the present investigation it has been treated merely as a chemical indicator. The study of hydrolysed, Giemsa-stained preparations has usually been much more fruitful.

It is difficult to correlate the strangely complicated and very irregular nuclear structures which Hollande and Hollande (1932) and Hollande (1934) have described in various spore-forming and non-sporing bacteria, with the dumbbell bodies of this investigation. In the absence of data on the results of the Feulgen test and of photographic evidence in support of the two authors' schematic, yet amazingly intricate drawings, no definite opinion on the significance of their observations can be formed.

Why treatment with hydrochloric acid should be necessary for the satisfactory demonstration of the chromatinic bodies of *B. mycoides* and other organisms is not clear, and naturally raises the question whether the chromatinic bodies are merely artefacts. This, however, is extremely unlikely for several reasons. Firstly, the chromatinic bodies may also be demonstrated by differentiation of Giemsa-stained films with 1 % watery solution of eosin. (I can confirm this discovery of Badian's (1933) for *B. mycoides*, though I find the chromatinic bodies much better defined in hydrolysed preparations.) Secondly, Bauer (1932) has shown that hydrolysis is a very gentle treatment and does not alter the size and shape of suitably fixed chromosomes. Finally, the fact that, like chromosomes, the chromatinic bodies of bacteria are constant in appearance and distribution and pass through a regular series of changes, strongly suggests that they represent an actual component of the living cell. Observation of living bacteria has so far been inconclusive. Further attempts in this direction are being made.

The cytological evidence, therefore, leaves little doubt that the chromatinic bodies represent a true nuclear structure. The actual form of the nuclear apparatus and its relation to the cell, as described in this communication, is rather different from the pictures presented by earlier work. Thus Stille (1937) and Piekarski (1937) describe the young vegetative form as a single cell containing two round chromatinic bodies, whereas my own results suggest that what these authors regard as a single cell is really a two-celled filament and that each chromatinic body actually consists of two closely contiguous, dumbbell-shaped structures. These authors' four-granule stage, which, according to their scheme, results from the simultaneous division of the two 'nucleoids', appears to be either a two-celled filament, or a four-celled filament about to break into two.

The presence in spores of Feulgen-positive structures has previously been recorded by Pietschmann and Rippel (1931), Milovidov (1935), Stille (1937) and Schaede (1939), but, with the exception of Stille, these authors mistook the faintly stained cytoplasm for the whole spore and failed to realize that both cytoplasm and Feulgen-positive body are enveloped by the spore membrane which is invisible in Feulgen preparations. The crosses and asterisk forms, which Allen *et al.* have recently described in the germinating spores of a bacillus isolated by them, presumably correspond with the chromatinic configurations I have described in *B. mycoides*. The bacillus studied by Allen *et al.* was obtained through the courtesy of Dr Appleby, and the resting and germinating spores and vegetative cells of this organism were found to contain essentially the same chromatinic elements as *B. mycoides* (figure 14, plate 9 and figure 40, plate 11). I cannot agree with Allen and his collaborators, however, about the necessity for invoking a meiotic process in their interpretation.

The bodies which Badian describes in *B. mycoides* and *B. subtilis* are obviously identical with the dumbbell-shaped structures recorded in this communication (cp. Badian's text-figure 1 and my figures 34, 35, plate 10), and their behaviour, positive Feulgen reaction and general staining properties make Badian's view of their chromosomal nature most plausible.

Treating the fixed cells with hydrochloric acid not only gives clearer results than Badian's method of differentiation of Giemsa-stained cells with eosin, but has a wider application. Thus it has disclosed the presence of a chromatinic body in the resting spore and made it possible to follow its transformation throughout the entire germination process.

The chromosome-like structures of bacteria differ in important aspects from the nuclei and chromosomes of more highly organized forms of life. Their position outside the cytoplasm of the spore, their temporary invisibility during spore germination, the almost complete disappearance of the chromatinic bodies from the cells after 24 hr. incubation on nutrient agar and their reorganization after sub-culture, are features which, so far as I am aware, are without parallel in the nuclear cytology of higher organisms.

SUMMARY

The structural unit of the nuclear apparatus of aerobic, spore-forming bacteria is a dumbbell-shaped body, giving a positive Feulgen reaction, and possessing a strong affinity for nuclear dyes.

Resting spores contain one of these bodies, closely attached to, but distinct from, a rod of non-chromatinic protoplasm. Division forms of this body occur in a few spores.

When germination begins, the dumbbell body enters the rod of protoplasm, where it soon becomes invisible.

When it reappears at a later stage of the germination process, it has divided into two closely contiguous dumbbell bodies.

Further divisions of the chromatinic bodies precede the divisions of the vegetative cells in a regular way. Each vegetative cell usually contains a pair of dumbbell bodies.

Similar chromatinic bodies have been demonstrated in the cells of *Proteus vulgaris* and two Sarcinae.

The two single Feulgen-positive bodies described by previous authors in the resting cells of various spore-forming and non-sporing organisms thus appear to consist of two pairs of dumbbell bodies, and the single 'cells' to which these writers refer are in fact short, two-celled filaments.

It is concluded that the dumbbell bodies are comparable to the chromosomes of plant and animal cells.

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EXPLANATION OF PLATES 9–12

Plate 9

All figures are magnified 3200 times. Except where otherwise stated preparations were fixed in osmic vapour and 70 % alcohol and treated with N/HCl before staining.

FIGURE 1. Resting spores of *Bacillus mycoides* 1110. Sublimate alcohol; iron haematoxylin. A delicate membrane protrudes over the poles of the spore to the left of the asterisk.

FIGURES 2–6, 9. Resting spores of *B. mycoides* 1110 with single or divided peripheral chromatinic bodies. Giemsa. Division forms in figures 2–6. The chromatinic body of the spore to the left of the asterisk is lying across the spore's cytoplasm. Change of focus reveals that its position is on top of, not inside the cytoplasm. See also figure 46, plate 4 for normal resting spores of *B. mycoides* 1110.

FIGURES 7, 8, 11. Resting spores of *B. mycoides* 16. Giemsa. Chromatinic bodies clearly separate from the non-chromatinic rod.

FIGURE 10. Resting spores of *B. mycoides* 1110. Feulgen, counterstained with light green. Two rings form in the left half of the picture.

FIGURE 12. Resting spores of *B. mesentericus*. Iron haematoxylin.

FIGURE 13. Resting spores of *B. mesentericus*. Giemsa. In the second spore from the left a chromatinic body is actually lacking, in the fourth it is merely out of focus.

FIGURE 14. Chromatinic double granule superficially attached to boat-shaped cytoplasm in resting spore of the 'aerobic bacillus from grass' described by Allen *et al.* (1939). Giemsa.

FIGURE 15. Germinating spore of *Bacillus* '*Soil* 2' (Howie and Cruickshank 1940). Giemsa. Spore membrane visible. For explanation see page 313.

FIGURE 16. Spores of *B. mycoides* 16. Incubated for 1½ min. with nutrient broth at 37° C. Giemsa without previous treatment with HCl. One spore has already acquired an affinity for the stain, a second one is preparing to.

FIGURES 17–19. Spores of *B. mycoides* 16. Incubated as previous preparation. Giemsa. Figure 17 shows swelling of some of the chromatinic bodies, and ring formation. Arrows indicate in figure 18 a swollen chromatinic body, in figure 19 a chromatinic ring in the process of entering the non-chromatinic rod.

FIGURE 20. Arrested germination in spores of *B. mycoides* 16. Chromatinic dumbbell bodies entering the non-chromatinic rods. Incubated for 1½ hr. Giemsa. Explanation in the text, p. 309.

FIGURE 21. Spores of *B. mycoides* 1110. Incubated as in figures 16–19. Giemsa. Arrow indicates a chromatinic ring in the process of entering the non-chromatinic rod. This cell is comparable with the dark cell just above the centre of figure 17 where the chromatinic ring, although clearly resolved in the preparation, is not recognizable in the photograph.

Plate 10

Germinating spores and young vegetative cells of *B. mycoides* 16. All figures are magnified 3200 times. Figures 22–27 incubated with nutrient broth at 37° C. Figures 28–37 incubated on nutrient agar at 37° C. Minutes give the time of incubation. Osmic vapour, 70 % alcohol, HCl, Giemsa's stain.

FIGURE 22. 2½ min. Chromatinic ring still visible in one of the spores, the remainder uniformly stained.

FIGURE 23. 5 min. Spores lacking visible structural differentiation.

FIGURE 24. 20 min. Reappearance of differentiation into chromatinic matter and faintly stained cytoplasm.

FIGURE 25. 40 min.

FIGURES 26, 27. 60 min. Broad bands of chromatinic matter lying across the very faintly stained cytoplasm.

FIGURE 28. 90 min. Pairs of chromatinic dumbbell bodies in two young vegetative cells.

FIGURE 29. 90 min. Division of chromatinic structure.

FIGURE 30. 75 min. (from the same series of films as figures 22–27). Cell on the left: incomplete division of chromatinic structure. Cell on the right: completed division. Note delicate cell boundary (arrow). Stained in the usual manner this 'two-cell filament' would appear uniformly stained and be regarded as a single cell.

FIGURES 31, 32. 90 min. Nearly completed division of chromatinic structures in two-celled filaments (2nd division).

FIGURE 33. 90 min. Advanced stages of division of chromatinic structures in two- and three-cell filaments. Single cell with broad chromatinic structure of the type shown in figures 26, 27, near lower edge of picture.

FIGURE 34. 2 hr. The four-cell filament in the upper half of the picture has broken into two two-cell filaments. Division of chromatinic structures in two-cell filament in right-hand bottom corner.

FIGURE 35. 2½ hr. Lying parallel and inclined upwards towards the left are three pairs of two-cell filaments arising from recent division of four-cell filaments. * breakage of a filament at the three-cell stage, i.e. after only one cell of the two-cell stage had completed its division.

FIGURE 36. 90 min. V-shaped chromatinic figures (stage preparatory to figures 31, 32).

FIGURE 37. 90 min. V-figures and dumbbell bodies. Cell boundaries uncertain (four-cell filaments?).

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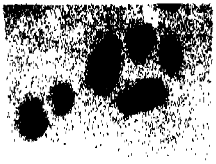
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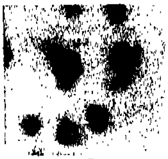
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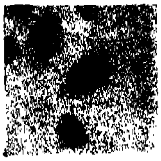
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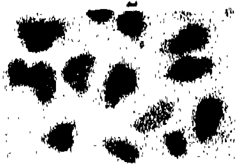
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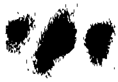
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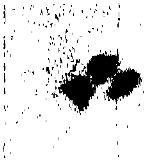
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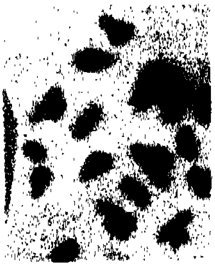
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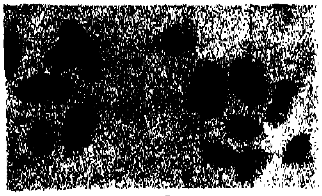
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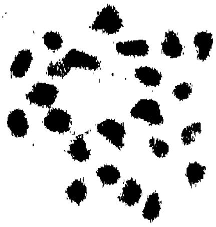
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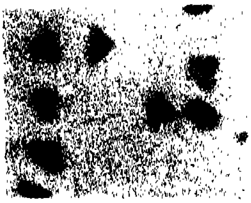
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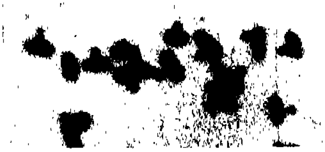
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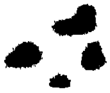
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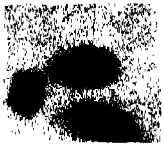
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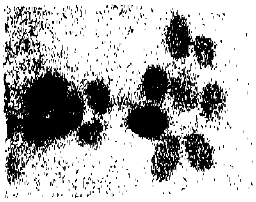
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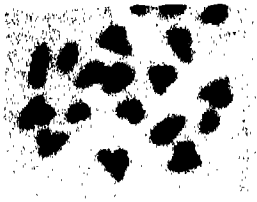
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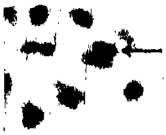
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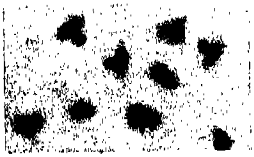
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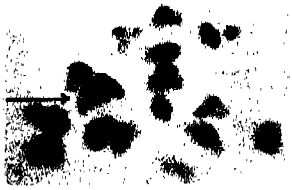
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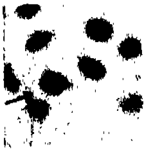
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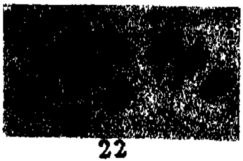
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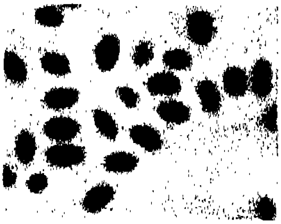
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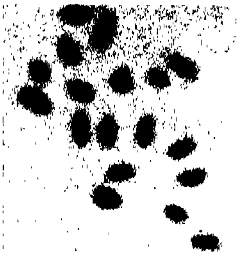
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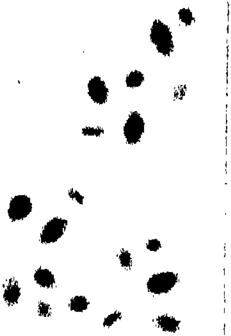
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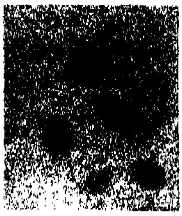
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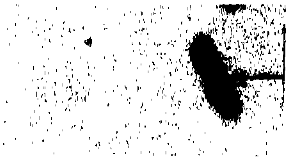
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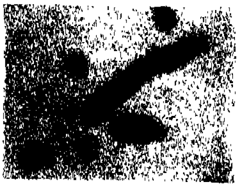
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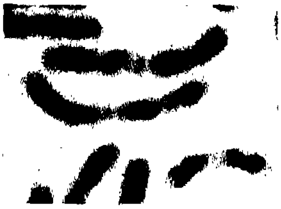
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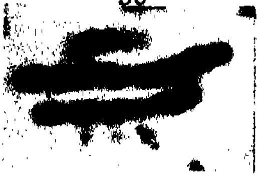
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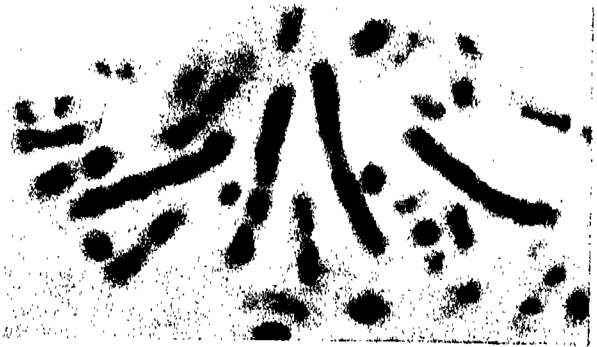
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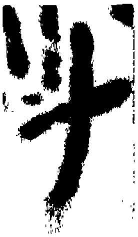

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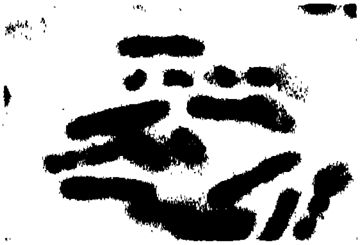

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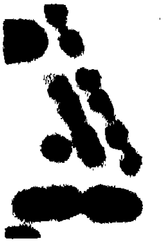

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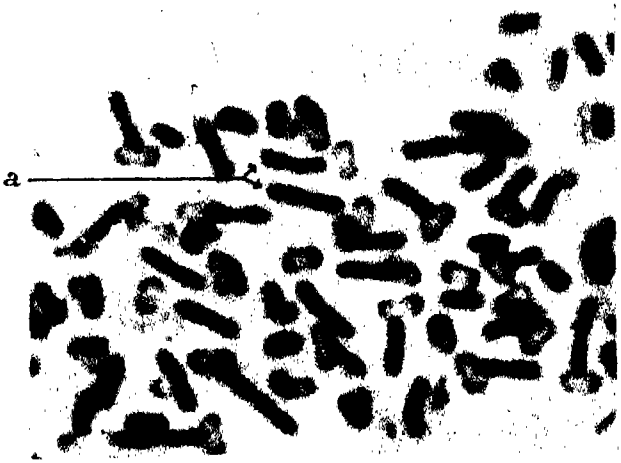

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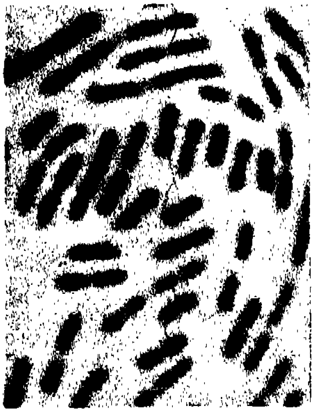

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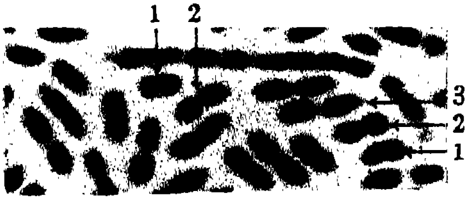

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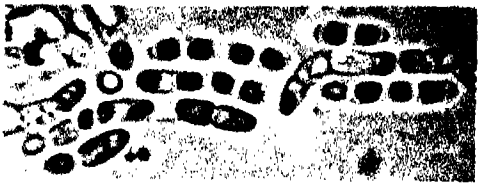

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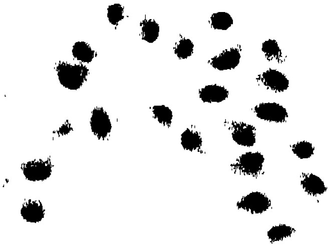

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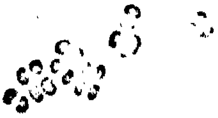

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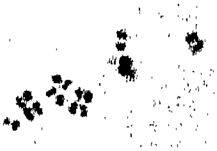

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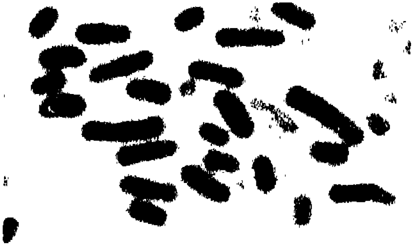

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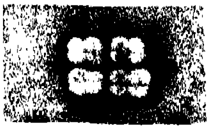

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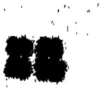

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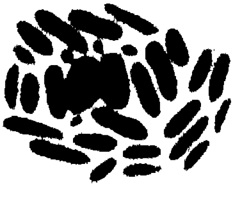

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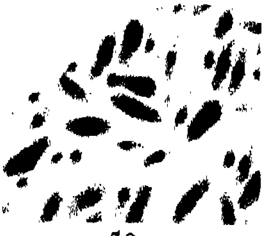

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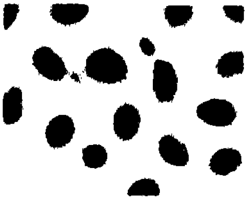

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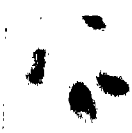

51b

51c

Plate 11

All figures are magnified 3200 times. Hours give time of incubation on nutrient agar at 37° C. Osmic vapour, 70 % alcohol, N/HCl, and, where not otherwise stated, Giemsa's stain.

FIGURES 38, 39. *B. mycoides* 1110. 1½ hr. Note delicate cell boundary in figure 38, separating the two upper pairs of dumbbell bodies in a four-cell filament. The shadow at the lower end of the filament is caused by the spore-case.

FIGURE 40. Aerobic spore-forming bacillus described by Allen *et al.* (1939). Four hours after subculture from 10-day-old colony on nutrient agar. Well-defined dumbbell bodies in two-cell filament. Note oblique cell boundary.

FIGURE 41. *B. mesentericus.* 2½ hr. Compare with figures 31–35 regarding distribution of chromatinic matter. Agar fixation method. Filaments marked 'a' correspond to figures 31, 32. Most filaments have not yet discarded their spare-cases.

FIGURE 42. *B. mesentericus.* 2½ hr. Feulgen reaction. No counter-stain.

FIGURES 43–44*b*. *Proteus vulgaris.* 6 hr. Fig. 43, agar fixation method. Slender rods containing 'Nucleoids' (cf. Piekarski 1939). Figures 44*a*, *b*, impression preparation. 'Nucleoids' resolved into pairs of dumbbell-shaped chromatinic bodies. Numbers indicate successive stages of division.

Plate 12

FIGURE 45. Spore formation in *Bacillus megatherium.* From a 2-day-old culture on potato agar. Osmic vapour. Photographed unstained in water. The number of spores pro-filament indicates the subdivision of the large rods of this organism into small, nearly cubical cells (de Bary 1884), and their distribution corresponds to that of the chromatinic structures in young vegetative forms of this and related organisms. (Compare with figures 33–35 of *B. mycoides.*) Magnification approximately 2700.

FIGURE 46. Resting spores of *B. mycoides* 1110. Osmic vapour, 70 % alcohol, N/HCl, Giemsa. Mag. 3200 times.

FIGURE 47. *B. mycoides* 16, grown from spores. 2 hr. on nutrient agar at 37° C. Osmic vapour, 70 % alcohol, N/HCl. Treated for an hour with 5 % watery solution of tannic acid at 37° C, then Giemsa. The differentiating effect of the HCl has been counteracted. Note two-cell filaments. Mag. 2100 times.

FIGURE 48. *Sarcina aurantiaca.* Two days on nutrient agar at room temperature. Osmic vapour. (*a*) Unstained in water. (*b*) Feulgen reaction of the same group of cells. Mag. 1800 times.

FIGURE 49. *Yellow sarcina.* Five days on nutrient agar at room temperature. Osmic vapour. (*a*) Unstained in water. (*b*) and (*c*) Feulgen reaction of the same group of cells, seen at two different levels of focus. A pair of Feulgen-positive granules visible in many cells. Mag. 1800 times.

FIGURE 50. *Torulopsis bacillaris.* Three days on Sabouraud agar at room temperature. Osmic vapour. (*a*) Giemsa without hydrolysis. Nuclei hardly differentiated from the cytoplasm. (*b*) Hydrolysis, followed by Giemsa. Nuclei well differentiated. There is one division stage. Mag. 2100 times.

FIGURE 51. *Monilia albicans.* Two days on Sabouraud agar at room temperature. Osmic vapour. (*a*) Giemsa without hydrolysis. (*b*), (*c*) Hydrolysis, followed by Giemsa. Two stages in the formation of a bud and beginning of nuclear division. Approx. mag. 1800 times.

Postscript. Owing to present conditions I have not yet been able to see a further paper by Piekarski (1940), *Arch. Mikrobiol.* **11**, 460, on positive Fuelgen 'nucleoids' in the resting and germinating spores of *Bacillus mycoides*. As far as could be ascertained, its contents confirm, but represent no significant advance on, the results of Stille (1937).

For the same reasons a recent well-documented description of nuclear structures in *Proteus vulgaris* by Neumann, F. (1941), *Zbl. Bakt.* Abt. II, **103**, 385, only came to my notice when the present paper was already in the press. Using Giemsa's stain and Feulgen's method, this author obtained clear pictures of homogeneous 'nucleoids' and in some cases of chromatinic dumbbell bodies. There is thus good agreement between his findings and those of the present investigation, but differences of interpretation are apparent in the two accounts.

Heterochromatin in *Triton*

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(*Communicated by C. D. Darlington, F.R.S.—Received* 8 *August* 1941)

[Plate 13]

When subjected to low temperature, certain segments of the chromosomes of *Triton vulgaris*, *T. palmatus* and *T. cristatus* are heterochromatic. At mitosis these segments are undercharged and at meiosis uncharged with nucleic acid.

These segments show the same type of allocyclic behaviour as do similar segments in *Paris*, *Trillium* and *Fritillaria*. They form Feulgen-positive chromocentres in all diffuse nuclei except pachytene, which is diffuse in *Triton*.

The availability of nucleic acid at the stage when the chromosomes normally spiralize is now shown to be a condition of that spiralization. The diffuse pachytene without chromocentres is followed by meiosis with unspiralized heterochromatin: the diffuse resting nucleus with chromocentres is followed by mitosis with spiralized heterochromatin.

With certain exceptions heterochromatin seems to be confined to those parts of chromosomes where chiasmata and crossing-over rarely occur.

1. INTRODUCTION

Longitudinal differentiation of the chromosomes is a condition underlying genetic analysis. This differentiation is visible equally at the pachytene stage of meiosis and in the salivary-gland chromosomes of Diptera. The typical chromosome at metaphase, however, is optically homogeneous. We know that this anomaly results from certain adaptations concerned with cell division. The chromosomes spiralize and become charged with nucleic acid preparatory to their anaphase movement on the spindle (Caspersson 1937, Summary; Darlington 1935; Darlington and Upcott 1939; *et alii*). Centromeres and nucleolar organizers are frequently visible because they do not behave like the other parts of chromosomes.

Since the work of Henking (1891) on the Hemipteran *Pyrrhocoris*, it has been known that the sex chromosomes of many insects and mammals 'condense' precociously during the spermatocyte prophase and then stain with basic dyes as intensely as the autosomes do at first meiotic metaphase. This reaction was termed *heteropycnosis* by Gutherz (1907). Heitz in 1928 introduced the morphological term *heterochromatin* to describe a particular kind of 'chromatin' which was distinguishable from another kind of chromatin, *euchromatin*, in its staining properties at telophase and prophase of mitosis. He at first thought that heterochromatin was specifically concerned with sex, but this view has since been discarded.

The problem of this reaction and substance has been extended and clarified by the work of Darlington and La Cour (1938, 1940, 1941). They find that certain regions of the mitotic chromosomes of species of *Paris*, *Trillium* and *Fritillaria* which appear euchromatic at metaphase when the plants are kept at normal temperatures, are heterochromatic when the plants are kept at low temperatures. These regions exhibit the phenomenon of heteropycnosis in that they are over-condensed in resting nuclei. Darlington and La Cour (1940) have accordingly introduced the term *allocycly* to describe the specific reaction (heteropycnosis) of the specific substance (heterochromatin) in terms of spiralization and nucleic acid charging. Similar conditions have now been found to obtain in the newt, *Triton* spp.

2. MATERIAL AND METHODS

Mitoses were studied in the tail tips of larval *Triton vulgaris* collected and reared at Merton. Adult males of *Triton vulgaris*, *T. palmatus* and *T. cristatus*, collected in a pond on Ranmore Common, Surrey, in the spring of 1940, supplied meiotic material. These males were placed on damp moss after the breeding season, and their testes were fixed during the early summer.

Acetic alcohol was found to be the most suitable fixative for *Triton*. Material was subsequently stained in iron aceto-carmine or by means of the Feulgen technique, tapped out on a slide and covered. Slides were made permanent according to the technique of La Cour (1937). The Feulgen technique was used only for mitotic material and as a test for nucleic acid. The hydrolysis involved in this technique causes the huge meiotic chromosomes to soften and contract, whereas with aceto-carmine they are well preserved.

Tail tips were examined untreated, after immersion in colchicine solution, and after exposure to low temperatures. It is difficult to analyse metaphase plates in the untreated larvae because the spindle is narrow and the chromosomes are crowded on its periphery. A few cells in larvae immersed in 1 % colchicine solution for 2 days were suitable for examination of the individual chromosomes, since the spindle is suppressed and the slightly super-spiralized chromosomes lie spaced out in the cytoplasm.

Temperature treatment, however, was the basis of manipulation. The minimum cold exposure to show heterochromatin is 3 days. Abnormal spindles arise in the mitotic cells of larvae exposed to 3° C (Barber and Callan, unpublished). The rate of entry of resting nuclei into mitosis falls off at low temperatures. It is thus rare to find analysable diploid complements which have reached metaphase spiralization while under the action of low temperatures. The only exposure which was found suitable therefore was one of 3 days' duration.

Testicular material was examined untreated and after exposure to low temperature. Temperatures of 3–6° C inhibit spindle formation in testis cells without preventing first spermatocytes at pachytene from reaching metaphase spiralization. Some material was fixed after 2–8 days of this treatment, but it was found that the bivalents were more easily examined when lying stretched on a spindle. This condition was realized by removing the newts from the refrigerator after 2 days at 6° C and then keeping them for 2 hr. at 18° C before fixing the testes.

By varying temperatures it is therefore possible to put the bivalents on or off the spindle as required for observation or experiment and at the same time control their spiralization and nucleic acid metabolism.

3. MITOSIS

The mitotic chromosomes of *Triton vulgaris* kept at 18° C are shown in the upper column of figure 1. The larva had been given colchicine treatment, consequently the chromosomes are somewhat super-spiralized and some have widely separated chromatids. There are six pairs of large chromosomes with median (*M*), two pairs of large chromosomes with submedian (*S*), three pairs of small chromosomes with submedian (*s*) and a single pair of small chromosomes with median (*m*) centromeres. Three pairs have secondary constrictions, two *M*'s and one *S*. Similar constrictions have been described by Parmenter (1920) in *Amblystoma tigrinum*, Janssens (1901) in *Triton* spp. and Fankhauser (1934) in *T. palmatus*.

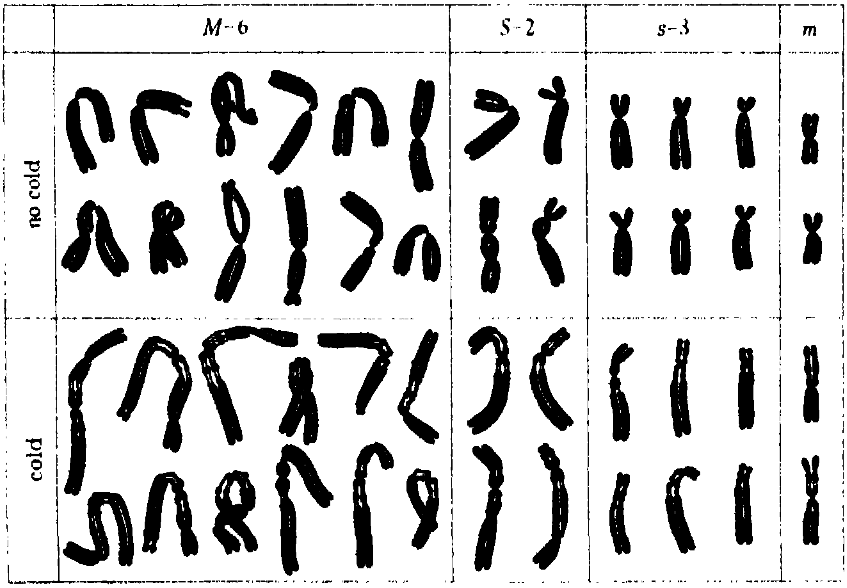

FIGURE 1. Mitotic chromosomes of *Triton vulgaris*. Feulgen. × 1200.

The mitotic chromosomes of a typical cold-treated larva are shown in the lower column of figure 1. It will at once be seen that the number of constrictions has increased enormously. So far as one can judge they are constant from cell to cell in one individual larva: this complement has 74. Almost all of the constrictions are crowded into the proximal regions of the chromosomes. Each constriction is very short unless stretched during preparation. Through it the deeply stained euchromatin segments are joined by a thinner under-stained strand just as in the plant material of Darlington and La Cour. The constrictions present in untreated material, and those induced by the action of low temperature are heterochromatic segments. They are deficient in nucleic acid and they produce a spiral of narrower diameter than that of the euchromatin.

They have the same type of allocycly as heterochromatic segments in plants. Thus resting epidermal nuclei have Feulgen-positive bodies lying in the region around the polar depression, i.e. close to the centromeres, which represent the over-condensed phase of the heterochromatic segments (figure 2).

The distribution of heterochromatin in *Triton vulgaris* closely corresponds to that found in *Fritillaria pudica* (Darlington and La Cour 1941). This plant species also has in the main short proximal segments.

The full development of under-stained regions, i.e. of nucleic acid deficiency, is first found in a few cells of larvae kept for 3 days at 3° C. Many cells are held at metaphase owing to anaphase suppression, but only those which have developed right through from the preceding resting stage in the cold give the complete expression. Cells which were in prophase when the treatment started show ill-defined heterochromatic segments. These are visible as slight constrictions, but they stain almost as intensely as euchromatin.

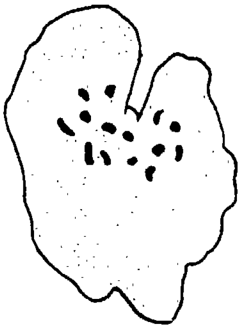

FIGURE 2. Resting nucleus of epithelial cell of *Triton vulgaris*, showing disposition of chromocentres around the polar depression. Feulgen. × 1800.

The effect of heterochromatin on anaphase can only be checked when larvae held at low temperatures for 3 or 4 days are released for an hour at room temperatures before fixation. A few cases of the sticking observed by Darlington and La Cour in *Paris* and *Trillium* (1938, 1940) were noticed. Other spindle abnormalities exist, however, and these make interpretation uncertain.

4. MEIOSIS

The normal meiotic chromosomes of *Triton vulgaris* and *T. palmatus* are shown in the upper columns of figures 3 and 4. The two species have generally similar complements. They differ in that *T. palmatus* has only one pair of large chromosomes which are markedly submedian (*S*), and no small median pair. Both species have distally localized chiasmata. *T. palmatus* has more extreme localization than *T. vulgaris* (Callan and Koller, unpublished).

While lying on the spindle at first metaphase, the bivalents stretch somewhat unevenly, giving slight indication of the position of the heterochromatin segments. Klingstedt (1936) has described similar constrictions in the normal meiotic chromosomes of the grasshopper *Chrysochraon*. The

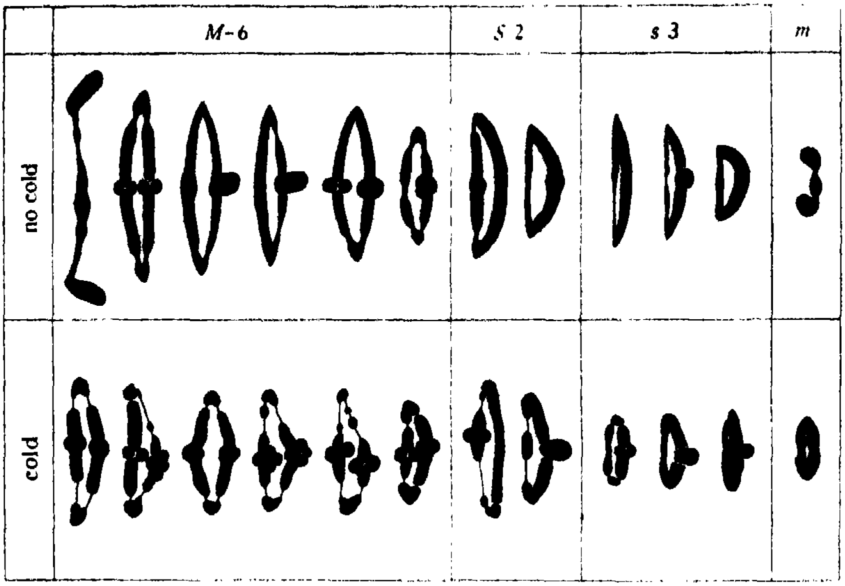

FIGURE 3. Meiotic bivalents of *Triton vulgaris*. Aceto-carmine. × 1200.

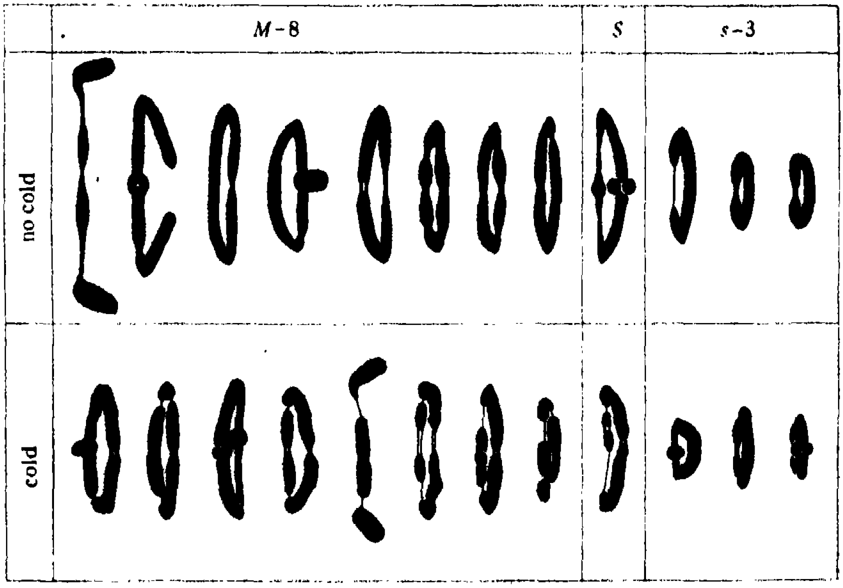

FIGURE 4. Meiotic bivalents of *Triton palmatus*. Aceto-carmine. × 1200.

heterochromatin interpretation might also be given to the constrictions seen at anaphase in the grasshopper *Stenobothrus* by Belar (1928, figure 266). Belar interpreted these constrictions as indicating places where the chromatids had broken at crossing-over.

In great contrast with this normal-temperature behaviour is that of the bivalents which have passed from pachytene to metaphase while under the influence of low temperature. Those shown in the lower columns of figures 3 and 4 have been subjected to 2 days at 6° C followed by 2 hr. at 18° C (see plate 13). As explained earlier, the meiotic spindle is inhibited at 6° C, and the short release before fixation was given in order that spindles should be formed.

The heterochromatin segments are often as clearly defined as they are in mitosis. They are completely Feulgen-negative. Each consists of a very thin non-staining strand between two blocks of euchromatin. Forty such segments are visible in the cell from *T. vulgaris*, and eighteen in that from *T. palmatus*. It is not possible to arrive at any conclusions about the constancy of regions from cell to cell, nor about possible hybridity. Only those segments which are seen from a suitable aspect can be identified with certainty. The segments are so short that in unstretched bivalents they are frequently obscured by nearby coils of the euchromatin spiral, and such segments resemble the constrictions between successive gyres. A greater proportion are rendered visible when the bivalents are stretched on a spindle, but one is never able to count as many segments at meiosis as at mitosis.

The unstained regions undergo great stretching in single-chiasma bivalents and in bivalents which are slow to separate at anaphase owing to their having formed more chiasmata than usual. Despite the fact that they are so much weaker than euchromatin in resisting longitudinal stress, no cells have been observed in which breakage has occurred. Nevertheless, it must be recognized that these segments constitute a source of weakness to the continuity of the chromosome thread, and that infrequent breakages at these points would provide the variation necessary for the play of selection.

Chromosomes at second meiotic metaphase also exhibit heterochromatic regions, if they have arisen from the preceding interphase under the action of low temperature. They are unsuitable for study, since they are small and greatly contracted.

The resting stage nuclei between spermatogonial divisions contain Feulgen-positive bodies similar to those in resting epidermal cell nuclei. The same is true of first and second spermatid resting nuclei. Pachytene is the last critical diffuse stage before first meiotic metaphase. This stage, unlike all other diffuse stages, does not contain nucleic acid aggregates.

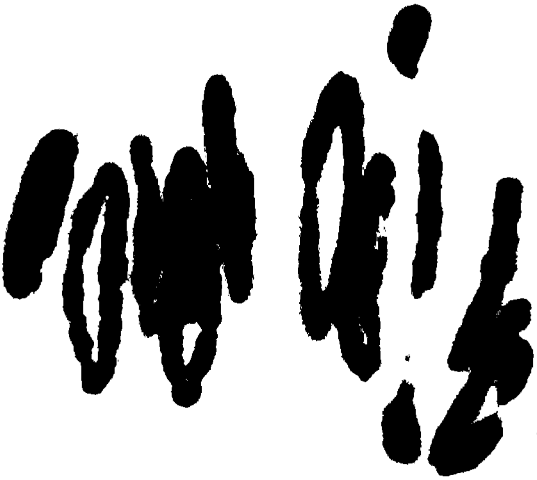

FIGURE 5. First metaphase of meiosis in *Triton palmatus*. 2 days 6° C, 2 hr. 18° C. Aceto-carmine. × 2000. Note stretched heterochromatic segments in *M* bivalent with single terminal chiasma.

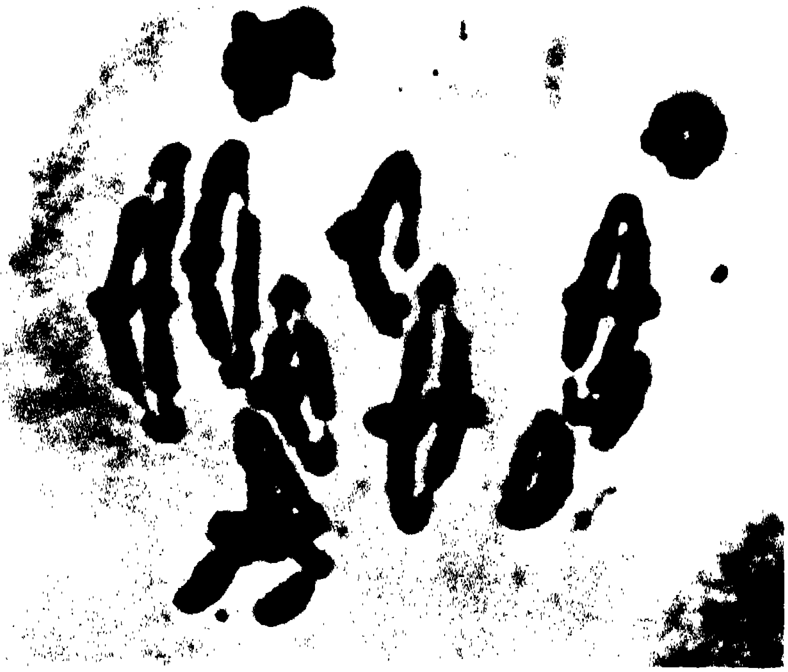

FIGURE 6. First metaphase of meiosis in *Triton vulgaris*. 2 days 6° C, 2 hr. 18° C. Aceto-carmine. × 2000. Note proximal heterochromatic segments in all orientated bivalents. The *S* bivalent in the middle of the plate may be heterozygous for a segment in the short arm.

It is clear from the examination of a few cold-treated meioses in *Triton cristatus* that this species also possesses proximal heterochromatic regions. It is, however, unsuitable material for a study of heterochromatin, since the first spermatocytes show much less localization of chiasmata than do those of *T. vulgaris* or *T. palmatus*. Chiasmata are formed in regions where heterochromatin is present also.

5. THE DETECTION OF HETEROCHROMATIN

Darlington and Upcott (1941) have suggested that the so-called 'inert' genes situated in heterochromatin segments are concerned with the metabolism of nucleic acid necessary for the efficient working of the cell cycle. If this is the case, we may presume that all organisms carry such inert genes, and we must ask ourselves what are the factors which determine that heterochromatin should be visible at metaphase in one organism and not in another.

Darlington and La Cour (1940) have discussed the phenomenon of allocyclic behaviour in terms of the 'supply and demand' of nucleic acid. Little need be added to their account. The detection of heterochromatin depends primarily on passing below the concentration threshold for the differential supply of nucleic acid during the prophase stages. Some organisms perform the natural experiment. Parts of the sex chromosomes of male Orthoptera and Mammalia, etc., show at certain stages and in certain cells the typical properties of heterochromatin. The same is true of certain individuals of *Mecostethus grossus* (Callan, unpublished) where proximal regions of certain of the autosomes behave as heterochromatin at meiosis, although they are not distinguishable at the metaphase of mitosis.

Low temperature is probably merely one of the agents which could assist in the detection of heterochromatin. When low temperature is the agent used, two conditions must be satisfied:

(*a*) A temperature must be applied sufficiently low to pass below the nucleic acid threshold, when the competition between genes for their supply of nucleic acid (this will be discussed under *Mecostethus*) results in the less 'active' genes obtaining little or none.

(*b*) The cell must be able to reach metaphase from a diffuse stage whilst under the influence of low temperature.

When an organism does not exhibit heterochromatin after a certain treatment, the conclusion to be drawn is not necessarily that it possesses no heterochromatin; one or other of these conditions may not have been satisfied. Thus from their work on root-tip mitoses of *Trillium*, Darlington and La Cour have shown that this plant possesses heterochromatin. Cold-treated pollen-grain divisions show no heterochromatin, however, owing to the enormous length of time between the previous diffuse stage and the pollen-grain metaphase. This period has never been covered by the treatment.

The newt provides very favourable meiotic material for the demonstration of heterochromatin. Its upper temperature limit is 6° C, at which temperature all processes but spindle formation continue. Also important is the fact that pachytene is a diffuse stage and lengthy treatment is thus unnecessary: only the pachytene-metaphase period needs to be covered.

6. HETEROCHROMATIN AND SPIRALIZATION

It has been suggested that heterochromatic regions do not have a spiral structure (Muller and Prokofieva 1935). White (1940) has shown that this view is manifestly inapplicable to the sex chromosomes of Orthoptera. Darlington and La Cour come to the same conclusion in their work on *Paris* and *Trillium* (1940) and in fact state that 'nucleic acid seems to have no necessary relation to spiralization'. The proximal heterochromatin segments in the autosomes of *Mecostethus* (Callan, unpublished) have approximately the same diameter of cross-section as euchromatin regions, and they may thus be considered to have the same type of spiral structure as euchromatin. Similarly, heterochromatin visible at mitosis in *Triton*, though of smaller diameter of cross-section than euchromatin, is considerably wider than the earliest prophase threads and thus probably has a spiral structure.

The heterochromatin visible in *Triton* at meiosis, however, is as thin as the leptotene or zygotene threads, despite fixation in acetic alcohol and staining in aceto-carmine, a technique which swells the chromosomes. When stained by means of the Feulgen technique, the tiny strands joining blocks of euchromatin are entirely unstained and in fact invisible. I am therefore convinced that neither minor nor major spirals exist in these regions. In the light of this evidence we must reconsider the relationship between nucleic acid and spiralization.

The sex chromosomes of Orthoptera spiralize while they are heavily charged with nucleic acid. If they become under-charged during meiosis, the loss of nucleic acid occurs during diakinesis and metaphase and *subsequent* to spiralization. The same is true of the autosomal heterochromatin in *Mecostethus*. At no stage are the heterochromatic regions of *Paris*, *Trillium* and *Fritillaria* completely negative to Feulgen, and we can thus presume that nucleic acid is always present during spiralization in these species.

During meiosis in *Triton* subjected to low temperature, the heterochromatic regions are completely negative to Feulgen throughout diplotene, diakinesis and metaphase, while in the diffuse pachytene stage there are no Feulgen-positive chromocentres. This fact and the absence of spiralization may be connected. We may in fact suppose that, while heterochromatin may or may not assume a spiral state, if it is utterly deficient in nucleic acid throughout the cell cycle, it cannot spiralize. This conclusion has important chemical and physical implications when we attempt to analyse the molecular mechanics of the chromosomes.

It is of interest to note that *Paris japonica* a species closely related to the *P. polyphylla* which has spiralized heterochromatin, has at mitosis thin thread-like regions very similar to those found in *Triton* at meiosis (Darlington and La Cour 1940). This argues against any major inherent difference between spiralized and unspiralized heterochromatin. It is a character of the cell, not of the segment.

7. HETEROCHROMATIN AND CHIASMA LOCALIZATION

Darlington has suggested that heterochromatin will develop in those parts of the chromosomes where chiasmata are rarely formed. When part of a chromosome fails to form chiasmata, recombination of the genes in this part is thereby prevented and these genes will finally mutate to inertness. The relationship between chiasma localization and heterochromatin regions is, however, complex (see table 1). *Paris polyphylla*, *Trillium* spp., *Adoxa moschatellina* (Geitler 1940), *Triton vulgaris* and *T. palmatus*, *Chrysochraon dispar* and the *Y*-chromosome of *Cricetus auratus* (Koller 1938) are examples which support Darlington's hypothesis. *Mecostethus grossus* is also no exception, since although both chiasmata and heterochromatin regions are proximally localized, chiasmata are never formed in the heterochromatin of this species. On the other hand, *Fritillaria pudica*, other *Trillium* spp., *Zea Mays* (Longley 1938; Darlington and Upcott 1941), female Orthoptera (McNabb 1928), *Triton cristatus* and *Drosophila melanogaster* are examples in which chiasmata are formed in the heterochromatin.

Darlington has put forward the further hypothesis that in such species as these the blocks of heterochromatin occurring in chiasma-forming regions are adapted to regulate the cross-over frequency in active regions nearby. The proximal heterochromatin in the *X*-chromosome of *Drosophila* is a case in point. It appears to be specifically adapted to achieve reciprocal cross-overs with the *Y* at meiosis in the male (Darlington 1934; Philip 1935).

TABLE 1. HETEROCHROMATIN AND CHIASMA DISTRIBUTION (EXCLUDING SEX CHROMOSOMES)

organism	initiation of pairing	chiasma localization	hetero-chromatin distribution	author
Paris polyphylla	procentric	intermediate	distal	Darlington and La Cour (1938)
P. japonica	,,	,,	intercalary	Darlington and La Cour (1940)
Adoxa moschatellina	,,	maximum	distal	Geitler (1940)
Trillium sessile	,,	intermediate	mainly distal	Darlington and La Cour (1940)
T. recurvatum	,,	,,	,, ,,	,, ,,
T. stylosum	,,	,,	,, ,,	,, ,,
T. grandiflorum	,,	,,	,, ,,	,, ,,
T. erectum	,,	,,	distal and proximal	,, ,,
T. kamtschaticum	,,	,,	,, ,,	,, ,,
Fritillaria pudica	,,	minimum	proximal	Darlington and La Cour (1941)
Mecostethus grossus	,,	maximum	,,	Callan (unpub.)
Triton palmatus	proterminal	maximum	proximal	Callan
T. vulgaris	,,	,,	,,	,,
T. cristatus	,,	intermediate	,,	,,
Chrysochraon dispar	,,	maximum	,,	Klingstedt (1936)
Zea Mays (with knobs)	,,	intermediate	distal	Longley (1938)
Zea Mays (with *B*-chromosomes)	,, ?	minimum	entire	Darlington and Upcott (1941)

Apart from any genetic inferences to be drawn from the distribution of heterochromatin and chiasmata, we must bear in mind that the mechanical properties of heterochromatin, its allocyclic behaviour, have some relation to the three prime variables of meiosis (Darlington 1940) which determine the distribution of chiasmata. In some organisms and under some conditions, heterochromatin is not allocyclic during meiosis. In these cases we have no evidence or a priori reason for supposing that heterochromatin behaves differently from euchromatin. In tetraploid spermatocytes of the grasshopper *Schistocerca*, the two *X*-chromosomes are allocyclic and form no chiasmata (White 1933). In the female, on the other hand, the two *X*-chromosomes are not allocyclic, and they form chiasmata like the autosomes (McNabb 1928). The heterochromatic *B*-chromosomes of *Zea Mays* which are allocyclic at meiosis form fewer chiasmata per unit length than the euchromosomes (unless the plant is trisomic for *B*'s when the 'triploid tie effect' counterbalances the mechanical properties of the heterochromatin (Darlington and Upcott 1941). In *Mecostethus grossus* the allocyclic proximal regions of the autosomes do not form chiasmata, and in fact the distance between the proximal chiasma and the centromere (the *differential* distance of Mather 1940) is precisely limited by the length of the intervening heterochromatic segment.

The analysis of the special problems connected with inert genes is only in its infancy. It is already clear, however, that by contrasting the properties of inert and active genes we have a further profitable line of approach to the general problems of heredity.

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THE CROONIAN LECTURE

The biology of epidemics

BY W. W. C. TOPLEY, F.R.S.

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Some, at least, among those who have shared with me the honour of being invited to deliver the Croonian Lecture must have shared also an uneasy feeling that they were under an obligation to refer, directly or indirectly, to some aspect of muscular motion, in accordance both with precedent and with the supposed intentions of the Founder. Uncertain of the facts, I consulted the Society's records, and found I need have no qualms. The Lecture was founded, not by Dr Croone, but by his widow, who, after his decease, married Sir Edwin Sadleir. It was founded by her for the Advancement of Natural Knowledge on Local Motion, or (conditionally) on such other subject as, in the opinion of the President for the time being, should be most useful for promoting the objects for which the Royal Society was instituted.

A Report on the Croonian Lecture, issued by the Society in 1834, states the position clearly and concisely. The relevant passage reads:

'The epithet *Croonian* which has hitherto (with few exceptions) been applied to this Lecture, instead of that of *Sadleirian*, to which in justice to its Founder it seems to be entitled, appears to have arisen in the misconception of the Council....Having mistaken Dr Croone as the Founder of the Lecture, they also mistook its nature and conditions; and instead of regarding it, as founded by Lady Sadleir, for promoting the general objects of the Society, they conceived it to be confined to the subject of Muscular Motion only; and the error, thus established, appears to have continued through subsequent years.'

In the light of this evidence, I am a little uncertain as to whether I am delivering a Croonian or Sadleirian Lecture; but I have no doubt at all that, without any dispensation from the President, I am entitled to discuss any phenomena depending, not on muscular, but on local motion. I can think of no better or more ambitious aim for any student of epidemics than to attempt to reduce his problems to precisely these terms. There is ample evidence that local movements of populations, of parasites and hosts, of insect vectors, and especially the relation of these movements to one another, determine outbreaks of infective disease. We are far as yet from identifying all relevant movements, or the factors on which they depend; and of those we know, many are hard to measure. But there is no other hopeful method of approach.

The epidemiology of to-day is the child of parasitology. Medicine is its grandmother, once or twice removed. The universe of study which now faces the epidemiologist is not, in its essence, composed of a number of cases of a clinically recognizable disease, with a distribution that varies in an intriguing way in space and time. It is composed of a variety of biological species, some acting as parasites and some as hosts. The parasites may be viruses, or bacteria, or fungi, or protozoa, or worms. The hosts may be men, or animals, or insects, or plants. Some parasites, particularly certain helminths, pass through different life cycles in different hosts, so that they are dependent on at least two host species for their continued propagation. Many parasites can propagate themselves in the tissues of two or more animal species which act as alternative hosts. Certain biting insects act as vectors. Sometimes the parasite passes through an essential phase of a complex life cycle within the insect's tissues. Sometimes it is simply transferred from the blood of one host to that of another, perhaps with multiplication by binary fission during transit. Sometimes non-biting insects act as mechanical vectors of parasites, commonly from excreta to food; but in these instances they provide only one among many possible routes of infection. Even if we take the simplest case in which there is no insect vector, and in which, so far as we yet know, only one host, say man, and one parasite are concerned, we find that our universe is not composed of sick persons and the parasite that causes the disease, but that there are, among the infected hosts, all gradations from clinically typical cases, through mild and atypical infections, to what are known as healthy carriers, persons who display no signs or symptoms of illness but from whose tissues or excreta the causative parasite may be isolated.

It is clear that no amassing of clinical observations, however careful and acute, and no correlation of such observations with environmental factors, however complete the records and statistical analyses, could have solved such problems as these, or have shown us in any detail how and when we might intervene effectively. Such observations have supplied us with data that form an essential part of our field of study; but when, to-day, we are faced with correlations between certain diseases and certain conditions of climate, or housing, or occupation, we do not leave it at that, we transpose our picture into terms of the effect of these environmental conditions on the hosts, or vectors, or parasites concerned. We think, for instance, not in terms of marshes and malaria, but in terms of breeding places for mosquito larvae, and of how these are affected by light, shade, salinity, and a host of other factors. We try to discover which mosquitoes bite which hosts and under what conditions, how far they fly, and where they hibernate, and when. We view the problem of plague in terms of rats and fleas, of typhus in terms of lice, of diphtheria in terms of carriers as well as cases, and so on. The medical or veterinary epidemiologist becomes a biologist whether he will or no. The biologist can study many of the problems of epidemiology with no more than a nodding acquaintance with human or veterinary medicine.

The very complexity of the natural systems with which the epidemiologist is faced often makes it difficult to tell which of the correlations that he observes are biologically significant. This difficulty is not lessened by the fact that it is quite easy to invent hypotheses that would, if they were true, fit attractively into our puzzle. It is much less easy to determine whether they are true or not; and this step has been omitted with a rather surprising frequency.

One way of attempting to solve some of our problems is to turn our backs on the natural world, and to simplify our conditions until the number of variable factors reaches manageable proportions, and then to see what happens when we hold some factors constant and vary others. This is the method that Professor Greenwood and I, together with our colleagues Dr Joyce Wilson and Dr Bradford Hill, have been exploring for many years past (Greenwood, Hill, Topley & Wilson 1936). We have worked with mice, and with three diseases to which mice are naturally prone: pasteurellosis, a bacterial disease of the respiratory tract; mouse typhoid, an intestinal bacterial disease; and a virus disease, ectromelia. All these diseases spread from mouse to mouse by direct contact. The various precautions that we have taken to control our experimental conditions have been described elsewhere.

THE EFFECT OF VARYING THE RATE OF ADDITION OF SUSCEPTIBLE MICE

In most experiments we have started an epidemic among a group of mice, and then added a constant number of mice each day for many months, or for several years. The effect of varying the rate of addition of susceptible mice to infected herds, contact being continuous, may be briefly summarized as follows.

With low rates of addition, up to one or two mice each day, the death-rate has shown wide and irregular fluctuations, with occasional intermissions. As the rate of addition rises, the curve of daily mortality assumes an undulating form, with no intermissions, and no clearly defined waves or peaks after the few initial fluctuations that always mark the earliest phases of epidemic spread under these conditions. The total population of the herd rises, at first steeply, then more slowly. In experiments carried on for many months or years it tends towards a relatively constant level. Figure 1 shows the first five months' experience in an epidemic of pasteurellosis, in which six mice were added daily to the herd. Fig. 2 shows similar periods for two epidemics of ectromelia, in each of which three mice were added daily to the herd.

It may be noted that, in our limited experience, there is little correlation between the average daily death rate and the rate of addition of susceptible mice, provided that immigration is maintained at a steady rate. It follows that the effect of adding more mice each day is simply to increase the level of population at which equilibrium is attained.

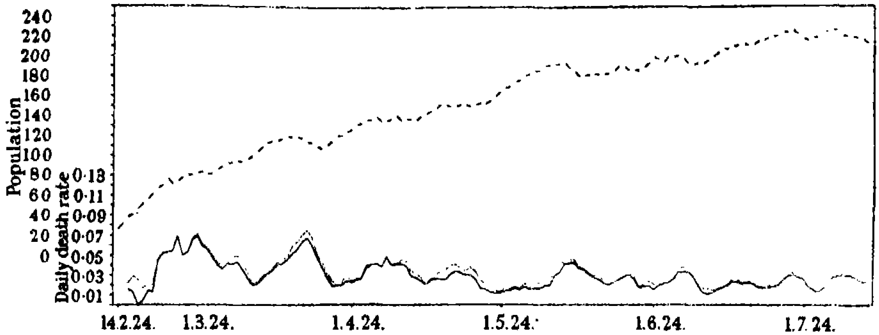


FIGURE 1. Secular death rate and population in the first five months of an epidemic of mouse pasteurellosis. Six mice added daily. - - - - - population: smoothed daily death rate (all deaths): —— smoothed daily death rate (specific deaths only).

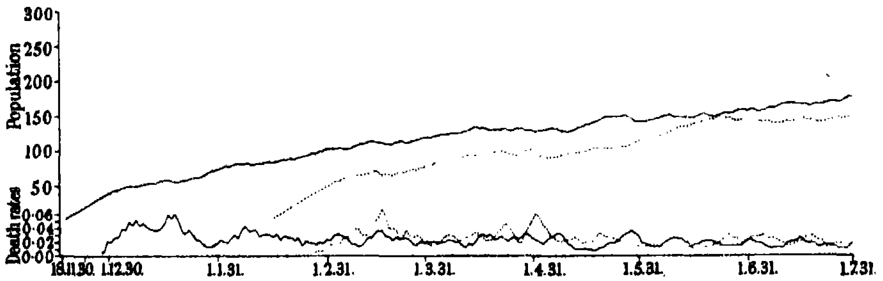


FIGURE 2. Two epidemics of ectromelia. Three mice added daily.

All our evidence suggests that, with rates of addition higher than have been practicable in our experiments, we should attain a steady rate of mortality and a stable population, the daily deaths equalling the daily additions. This means only that such a system has no inherent tendency to fluctuate, as epidemics fluctuate under natural conditions. There is no evidence that the parasite waxes or wanes in its relevant biological properties as it passes from host to host, or that the hosts pass through any periodic variation in resistance. Nor does the infection ever die out. We must search in other directions for the factors that determine the rise and fall of epidemic waves.

THE EFFECT OF CHANGING FROM CONTINUOUS TO DISCONTINUOUS CONTACT

In an experiment which has not yet been recorded in detail we varied the procedure described above by keeping the mice constituting an infected herd in separate cages, one mouse to a cage. We started the experiment with twenty-five mice infected with mouse typhoid and 100 normal companions. Each Monday, Wednesday and Friday the mice were assembled in a single large cage for 4 hrs, and on each of these days two normal mice were added to the herd. The course of events is shown in figure 3. The herd was assembled on 19 April 1937. An epidemic started, but soon died down; and after about the 70th day deaths from mouse typhoid ceased to occur. The population was rising, and by the 149th day had reached 180. Since no death from mouse typhoid had occurred for approximately 80 days, it seemed that these conditions, though involving close contact between the mice for 4 hrs on each of three days a week, were not such as to ensure an effective spread of infection. The conditions were therefore altered, and on the 149th day the mice were aggregated in a single large cage. The response was almost immediate. Within a few days

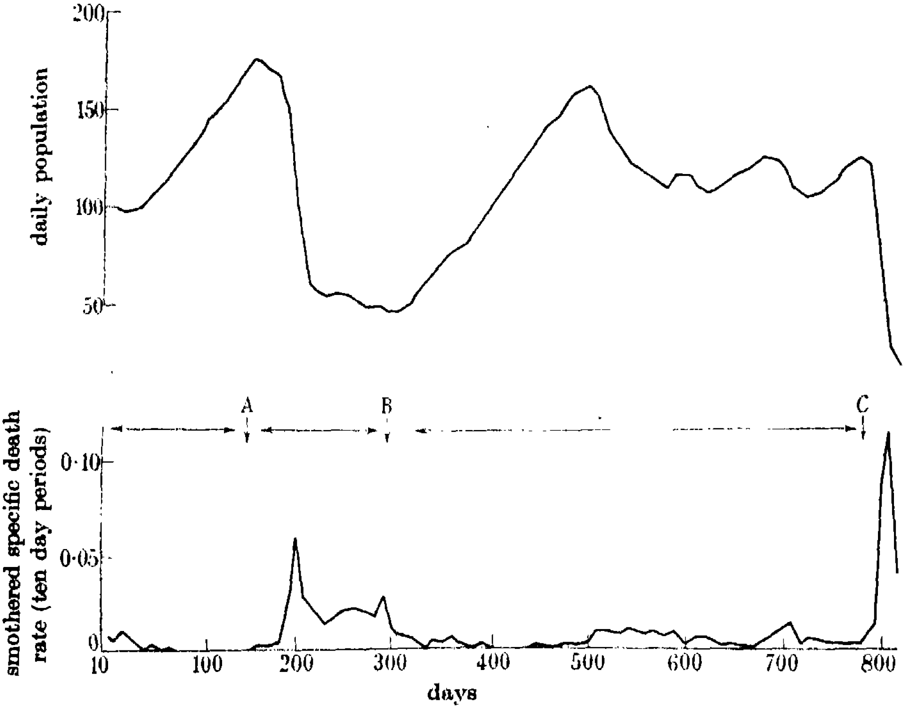


FIGURE 3. A, contact becomes continuous; B, contact becomes intermittent; C, contact becomes continuous.

deaths from mouse typhoid recurred, and, within 35 days of aggregation, a major epidemic wave was well under way, reaching a peak 15 days later. After this wave had subsided, the mortality remained at a relatively high level, and by the 289th day of the experiment the total population had fallen from 180 mice to 44, in spite of the addition of 6 new entrants a week. On day 290 the herd was again separated into single cages, and allowed to mingle for 4 hrs on each of three days a week. The response was as prompt as before, but in the reverse direction. The death-rate sank, and the population mounted. These conditions were maintained from the 290th to the 767th day, a period of over 1 year and 3 months. During this period the death-rate followed an irregular course. It seemed clear that intermittent contact of this degree, while it had been insufficient to maintain the initial epidemic spread when only a few of the mice were infected, was adequate to maintain a smouldering endemic prevalence after the massive infection of the herd that followed the first aggregation. On the 767th day the herd was aggregated in a single cage for a second time. Again there was a brisk response. A major epidemic wave started within 15 days, reaching a peak about 20 days later, and then fell sharply. Within 30 days the population fell from 150 to 20 mice. The experiment was shortly after brought to an end. Its interest lies in the striking response to simple changes in the continuity of contact without the introduction of any new source of infection.

THE EFFECT OF DISPERSAL ON AN INFECTED HERD

We have carried out a few experiments to test the effect of dispersing an infected herd into groups of varying size, no subsequent additions being made (Topley 1922; Topley & Wilson 1925). The results indicate that dispersal into relatively large groups, for instance the dispersal of 100 mice into four groups each of 25, has little effect once an epidemic has begun to spread. If the groups are smaller, if for instance, a herd of 100 mice is dispersed into 10 groups of 10 mice apiece, the subsequent specific mortality is much lower than in a control herd of 100 mice maintained as a single unit, provided that dispersal is carried out in the initial phase of an epidemic. If, on the other hand, dispersal is delayed until an epidemic wave is well under way it has little effect, even though the dispersed groups are numerous and small. The withdrawal of mice from a herd, in which infection is continuously spreading, to isolation in single cages, always lowers the subsequent average mortality of these mice as compared with that of their contemporaries left in the herd, however long they have previously been exposed to risk (Greenwood *et al.* 1939).

GROUP-TO-GROUP INFECTION

In the same category of experiments depending on the controlled movements of infected and susceptible mice in relation to one another, we may include a small series of tests designed to answer a rather different question (Topley & Wilson 1923). In the many experiments in which we have added normal mice to an infected herd for periods of months or years, the infection has never shown any signs of dying out. It was clearly of interest to see how long an infection could be propagated if a small group of infected mice was placed in contact with a small group of normal mice for a relatively short period, the normal contacts removed after this exposure and placed in contact with another group of normal mice, and this process repeated as long as infection continued to pass from group to group. The results of these tests were in sharp contrast to those obtained when normal mice were added to a herd in which infection was spreading continuously. By the group-to-group method of contact, the period of contact between groups varying from 4 to 21 days, it was never possible to secure the passage of mouse typhoid or of pasteurellosis beyond the third successive group. We have not yet made similar experiments with ectromelia. Had the groups been larger, or the period of contact between them longer, continuous spread would probably have been secured. These experiments are merely another illustration of the dominance in the spread of infection of those factors that determine the probability of effective contact.

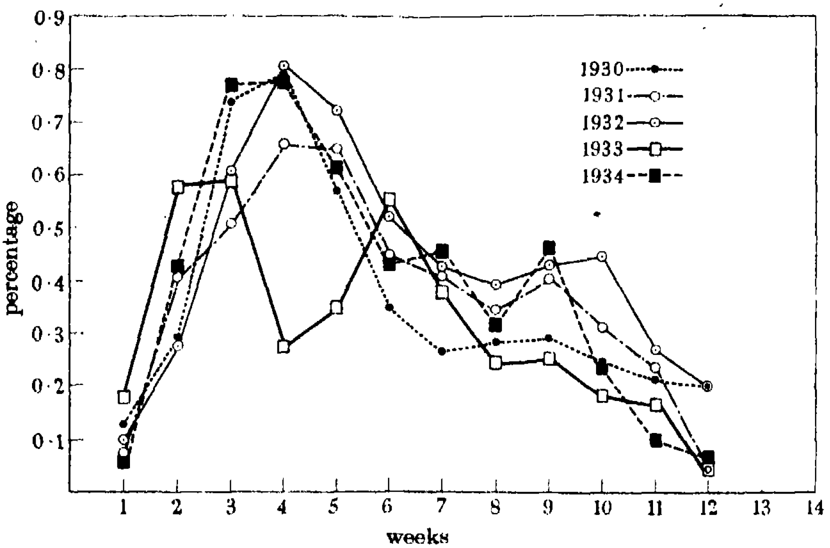


FIGURE 4. Showing weekly attack rates per cent of all nasopharyngeal infection (excluding influenza) for the five Lent terms 1930–4 (girls' boarding schools).

There is no difficulty in finding analogies to the events in our cages in the natural world outside them. As examples we may take some of the data collected by my colleague, Dr Joyce Wilson, on behalf of the School Epidemics Committee of the Medical Research Council (School Epidemics Committee 1938). They were obtained from twenty-one public schools for boys, and ten public schools for girls. Complete card records were made, for the five years 1930–4 inclusive, of all forms of sickness involving an absence from school on one or more days.

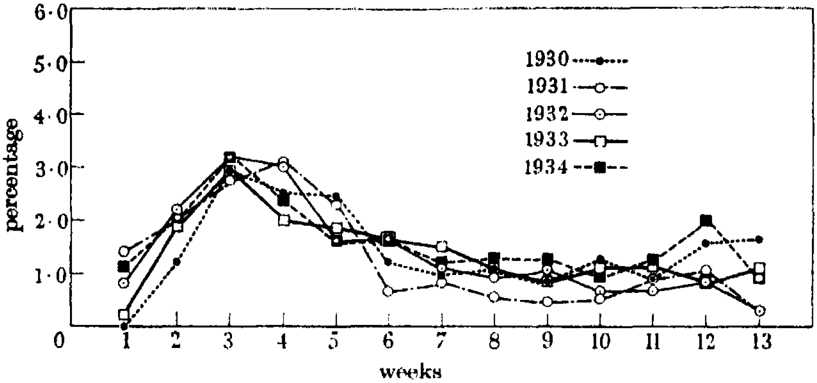


FIGURE 5. Showing weekly attack rates per cent of all nasopharyngeal infection (excluding influenza) for the five Summer terms 1930–4 (girls' boarding schools).

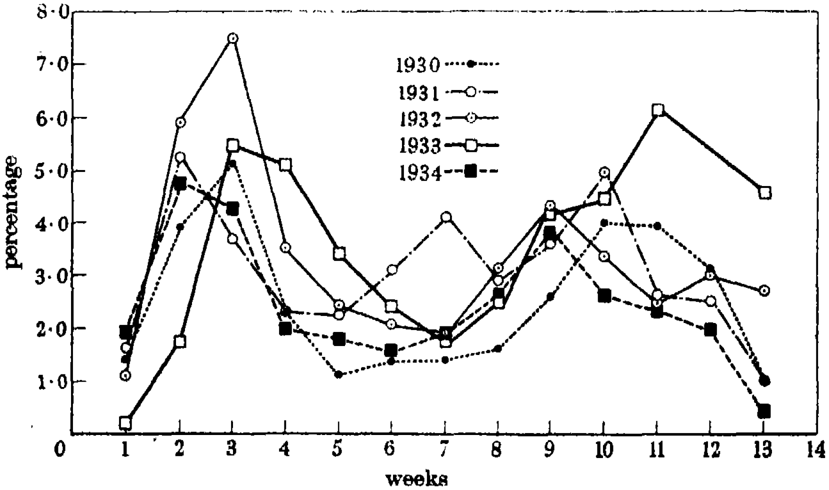


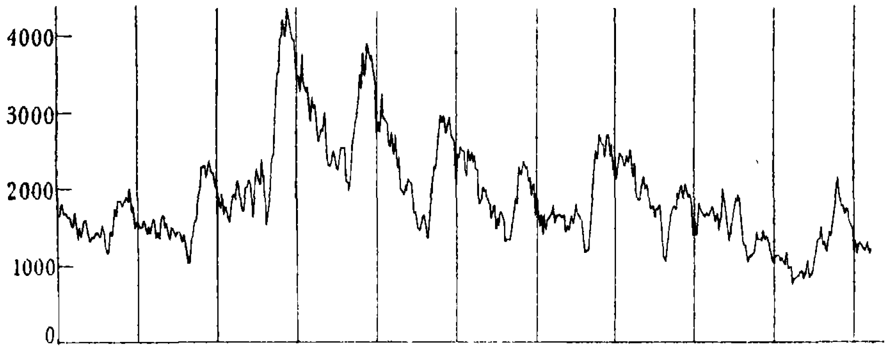
FIGURE 6. Showing weekly attack rates per cent of all nasopharyngeal infection (excluding influenza) for the five Christmas terms 1930–4 (girls' boarding schools).

Taking the group of minor nasopharyngeal infections, colds, sore throats and so on, figures 4, 5 and 6 show the attack rates for these disorders for each week of the Lent, Summer and Christmas terms in each year for the ten girls' schools. It will be seen that in every term, in every year, there was a primary peak incidence between the second and fourth week of term, usually between the third and fourth. Similar curves for any of the common infectious diseases, such as measles, tell an analogous story.

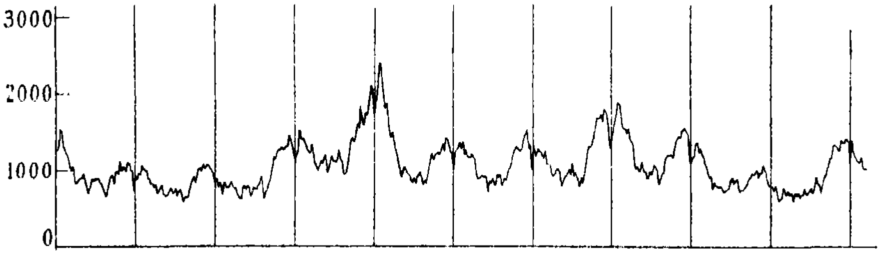
There can be no doubt that this termly distribution of infective disease is the direct result of the reaggregation of boys and girls after the dispersal of the holidays. It cannot be due to seasonal influences, since the same thing happens in each of the three terms, save that the peaks are higher in the Lent terms for those diseases that are prevalent in the late winter and early spring. Epidemics of this kind are, at the moment, part of the price that we must pay for education.

It is of some interest to trace the effect on these contact infections of the evacuation of children from our larger and more vulnerable cities which occurred on the outbreak of war. Figure 7 shows the weekly incidence of scarlet fever and diphtheria in England and Wales for the ten years 1931–41. For measles and whooping cough, which have only recently been made notifiable, the weekly deaths in the 150 great towns have been taken over the same period. The vertical lines are drawn at 31 December each year.

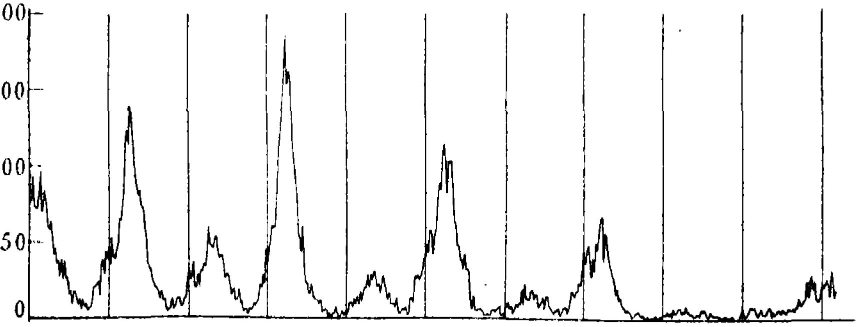
It is obvious at a glance that the last three months of 1939 and the greater part of 1940 were periods of unusually low incidence so far as these diseases were concerned; and the change in the form of the yearly curves, with the exception of that for diphtheria, is as striking as the total fall in the cases or deaths. Scarlet fever, in 1940, fell to its lowest level in the early spring, instead of in the late summer. The expected rise in the measles death-rate in the winter of 1939–40, and in the early spring of the latter year, is conspicuous by its absence; and the same is true of whooping cough during the early months of 1940. It might be argued that the abnormal curves for measles and whooping cough are due to the figures for these diseases being taken from the great towns. When the curve was drawn, monthly mortality figures for the country as a whole were not available; but Dr Stocks, of the General Register Office, has kindly supplied me with the relevant quarterly figures for the period in question, and these show that the number of deaths in England and Wales was so low that no epidemic can have occurred. There are, however, many considerations that have to be taken into account in interpreting secular curves of this kind; and it is possible that the decrease in any of these diseases would have occurred apart from the war. The cumulative evidence does, however, suggest that the association with evacuation was not in each case fortuitous. Assuming this, it seems at least possible that school closure in towns, and the lack of school accommodation in the evacuation areas, contributed more to the result than the move from town to country per se.



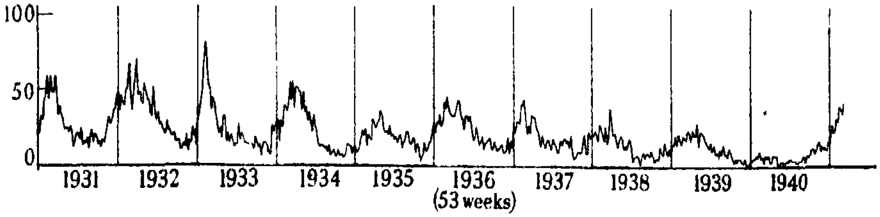
England and Wales, weekly notified cases of scarlet fever (1931–41).



England and Wales, weekly notified cases of diphtheria (1931–41).



Great towns of England and Wales, weekly notified deaths from measles (1931–41).



Great towns of England and Wales, weekly notified deaths from whooping cough (1931–41).

FIGURE 7

At the same time that we were evacuating children from our larger towns, and decreasing their contacts in school, we were collecting a large part of our young adult males into huts and billets. Young adults are, for the most part, resistant to scarlet fever, diphtheria, measles and whooping cough; but they are susceptible to another disease, cerebrospinal meningitis, which is also spread by contact infection by way of the upper respiratory tract. Whenever we mobilize an army we may expect an outbreak of this disease during the first winter or spring, when the men spend a large part of their time in huts or billets. In spite of the low rates of other sickness in the army, this particular expectation was fulfilled. Figure 8 shows that a major epidemic of cerebrospinal meningitis occurred in the early months of 1940. During the rest of the year the incidence remained higher than in any year of peace, and there was another epidemic wave, though on a smaller scale, in the early months of 1941. Fortunately, the new sulphonamide drugs had provided us, for the first time, with an effective remedy; and though the morbidity was high the case mortality was low.

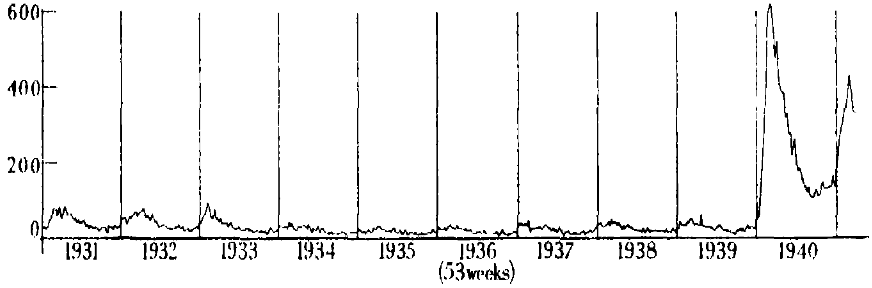


FIGURE 8. England and Wales, weekly notified cases of cerebro-spinal fever (1931–41).

The lesson to be drawn from this is, I think, the following. It is quite certain that movements of susceptible and infected hosts in relation to one another, and aggregations or dispersals of human or animal herds, apart from any introduction of new infection, are sufficient to induce major changes in the incidence of many infective diseases. In considering the relation of any environmental factors to the rise and fall of epidemic waves, it will always be wise to determine in what way they affect the movement and distribution of the hosts at risk.

Equally, when we attempt to lessen the incidence of an infective disease, it will be wise to consider carefully whether any practicable change in the habitual movements and distribution of infected and susceptible hosts will lessen the frequency of contact between them.

The same applies to the mechanical transference of parasites, through the air, by dust, by contaminated objects, by persons or animals who may transfer the parasites from host to host without themselves becoming infected, and so on. The system becomes more complex, the risks more numerous, and the necessary measures of control more various, but the principle does not change. Any step that lessens the probability of effective contact, direct or indirect, is a step in the right direction. This seems a platitude; and so it should be. But there are many instances in which the possibilities of action along these lines have not yet been fully exploited.

THE EFFECT OF NATURAL AND ARTIFICIAL IMMUNIZATION

In summarizing the results obtained in long-continued experimental epidemics, it was noted that there was no evidence of any periodic change in the resistance of the hosts at risk of a kind that would lead to fluctuations in the rate of mortality. This does not mean, of course, that the resistance of the mice is uniform on entry to the herd, or remains unchanged thereafter. The individuals of any host species vary in their innate resistance, and the average resistance of mice that have lived for long in an infected herd will be greater than the average resistance of mice on entry, if only because the more susceptible will have been sifted out by death.

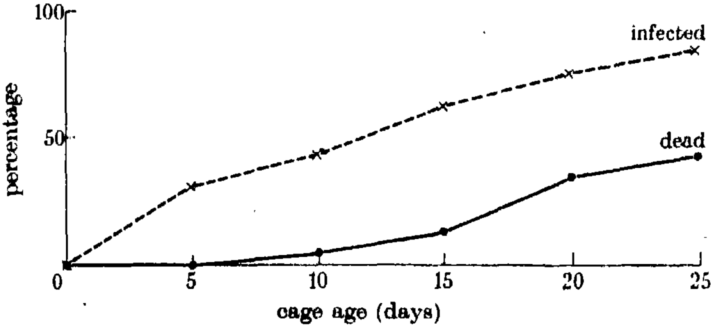


FIGURE 9. Percentage of infections and deaths among ninety mice exposed to mouse typhoid, for different cage ages.

Those who survive do not remain unchanged. The great majority of them react to a non-fatal infection by developing an immunity of the type that depends on the production, in the host's tissues, of antibodies that react specifically with certain constituents of the infecting micro-organisms.

It would be easy to give any number of illustrations of this type of immunization from the world of natural events, but the data available for infected herds of mice are more complete, and therefore more easily represented in graphic form. Figure 9 is constructed from the results obtained in a particular epidemic of mouse typhoid in which daily examinations were made of the excreta of all mice at risk, so that infection could be detected apart from death (Topley, Ayrton & Lewis 1924). The abscissae are days of cage age, not days in secular time. Mice dead of the disease are included among the infected, so that the difference between the broken and continuous curves represents mice infected but still alive. It will be seen that, by the 25th day of residence in an infected herd, 42 % of entrants are dead, and 41 % infected but alive. The data from many other epidemics indicate that infection rates of this order hold for most of our experiments, though there is naturally some variation. We can, at least, regard it as highly probable that most survivors of a few weeks' standing have acquired the specific infection, and with it the stimulus that we should expect to result in some degree of specific immunity. In considering how far our expectations are realized it will be convenient to select experiments in which the fate of normal entrants has been compared with that of mice

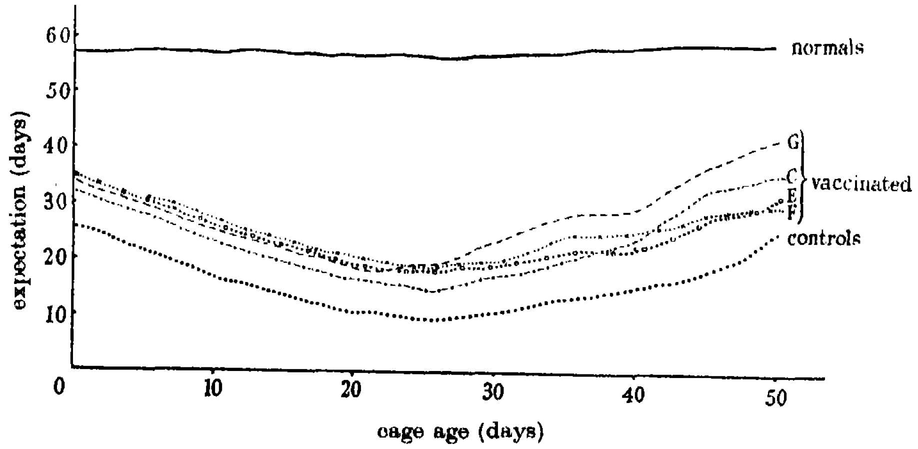


FIGURE 10. Effect of vaccination in mouse typhoid; expectation of life limited to sixty days.

artificially immunized with an appropriate vaccine, so that we may at the same time assess the extent to which we can gain the advantage of immunity without the risk of death.

Figure 10 shows the relevant findings in the case of mouse typhoid (Greenwood, Topley & Wilson 1931*a*, *b*). The ordinates give the average expectation of life arbitrarily limited to 60 days. Here again, the abscissae are days of cage age, not days in secular time. The upper curve shows the limited expectation of life at all days of cage age from 0 to 50 of normal mice living in an uninfected herd to which three normal mice were added each day. As one would expect, the expectation of life approximates closely to its maximum value throughout, though it is somewhat below it during the earlier days of cage life before the mutual antagonisms of new entrants and old inhabitants have been replaced by a reasonable degree of tolerance. The lower curves show the change in expectation of life with cage age of mice that were living, and dying, in a herd infected with mouse typhoid, to which groups of vaccinated and control non-vaccinated mice were added together at regular intervals. It will be seen that the controls fared badly. On entry they had less than half the normal expectation of life, and their expectation sank till about the 25th day, when it was only 9·3 days out of a possible 60. This fall is due to the fact that the expectation of life will be minimal on the day of cage age at which, on the average, the mice have become infected and are in the final stages of a fatal illness. The expectation of life then rises, in part because the more susceptible mice have been eliminated by death, in greater part, perhaps, because the survivors have been specifically immunized by a non-fatal infection.

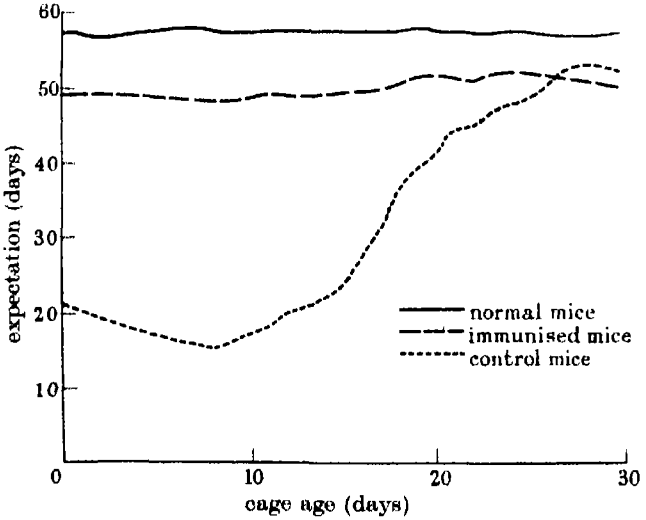


FIGURE 11. Effect of vaccination in ectromelia: expectation of life limited to sixty days.

The curves labelled *C*, *E*, *F* and *G* refer to mice artificially immunized by the injection of two doses of four different killed bacterial vaccines, all containing the essential antigenic constituents of *Bacterium typhi-murium*. It will be seen that, at all cage ages, they fare better than the non-immunized controls; but they never attain an expectation of life approaching the normal. It should, however, be noted that the mice in these experiments are exposed to a continuous risk of contact infection greater than would be encountered by any human population, except under very unusual conditions. The effectiveness of antityphoid inoculation in man has been amply proved by the experience in our armies.

Figure 11 shows similar curves for an epidemic of the virus disease ectromelia (Greenwood *et al.* 1936). The immunized mice in this instance were injected with two doses of a living but attenuated virus. The results here differ sharply from those obtained with mouse typhoid. On entry to the cage the control mice have an expectation of life even lower than that of the non-immunized entrants to the herd infected with mouse typhoid. But, after a slight fall, reaching its lowest point on the 8th day of cage age, the curve rises sharply, and by the 30th day has attained a value not far below the normal. Clearly, the natural immunization that follows a non-fatal attack of ectromelia is more effective than the natural immunization that follows an attack of mouse typhoid. Similarly, our vaccination has been far more effective. The immunized mice on entry to the cage have an expectation of life of almost 50 days out of a possible 60. The expectation never falls significantly below that figure, and later rises above it. We have placed our immunized immigrants, from the start, in the same position as that reached by the surviving non-immunized controls after some 20 days of cage life, when they have passed through an experience to which more than half the normal entrants have succumbed. It should be noted that, in this experiment, the limited expectation of life declined between the 30th and 50th days of cage life; but this decline applied equally to the control and vaccinated mice.

It is clear that the process of natural immunization will result in changes in the proportions of resistant and susceptible hosts among any community exposed to the risk of infection by a particular parasite. After the subsidence of a major epidemic wave the proportion of susceptibles will be decreased to a level depending on the average risk of infection to which the community as a whole has been subjected. If no new susceptibles gain access, an equilibrium may persist in which a high infection rate is balanced by a high level of herd immunity. If, after such an equilibrium has been maintained for a considerable period, a relatively large number of susceptibles gain entrance within a short interval of time, another major outbreak may occur, in which the newcomers suffer first and most severely, but in which some of the old survivors are also involved (Topley 1921). This sequence of events has been observed both in experimental epidemics and in natural outbreaks of disease. If, on the other hand, susceptibles gain access in small numbers, continuously or intermittently, the course of events will depend in the main on the average risk of infection to which the newcomers are subjected. If it is very high, there will tend to be a persistent endemic prevalence, with occasional cases of disease, but no major outbreak. If it is low, the proportion of newcomers at risk may attain a relatively high level before a chance succession of infections sets a spark to the susceptible material and precipitates a new epidemic wave.

It is probable that changes in the spatial distribution of the hosts at risk, and changes in the proportion of susceptible and resistant hosts resulting from natural immunization, together account for many, perhaps most, of the periodic or repetitive fluctuations in prevalence observed under natural conditions in those epidemic systems in which infection is spread by direct contact, and in which no insect vectors or alternative hosts are involved.

It should be noted that the effectiveness of natural immunization varies, both in degree and duration, from one infective disease to another, and that, apart from variations of this kind, the immunity involved is in each case specific, not to the clinical disease as such, but to the chemical constitution of the parasite that causes it, or of the toxin that the parasite produces. The chemical components concerned are known as antigens, and it happens that micro-organisms with different antigenic structures may produce indistinguishable symptoms and lesions in infected hosts, so that an acquired immunity towards a particular species or type of parasite may not be synonymous with immunity to the clinical disease of which it is one among several causes. There are, for instance, more than thirty different antigenic types of pneumococci that cause pneumonia in man, several types of the virus that causes foot-and-mouth disease in cattle, and, very unfortunately, more than one type of virus that causes human influenza.

We may now turn to factors of a different kind, which come into play at certain times, and in certain places, rather than as constant contributors to the course of events in any prolonged prevalence of infective disease.

THE EPIDEMIC POTENCY OF DIFFERENT STRAINS OF A SINGLE SPECIES OF PARASITE

Many field epidemiologists, and particularly the late Dr Fred Griffith, have been convinced that the observed behaviour of certain human infections, such as those due to haemolytic streptococci, demand the hypothesis of the existence of special epidemic strains of the parasites concerned, with heightened powers of producing disease by contact infection.

In an attempt to test this hypothesis experimentally, we have carried out a large number of trials by a method which differs from that employed in our long-term epidemics. We have assembled 100 mice in a single cage, and added to them 25 mice infected by the injection of a constant dose of the strain of bacterial parasite under test. We have watched events for 60 days, and then killed all surviving mice. We should not expect that experiments of this kind, even though carried out with a single strain of a particular bacterial parasite, would give us consistently replicable results, nor do they do so; but the variations are not so wide as might be feared.

Figure 12 shows the course of events in thirteen such epidemics, the number of survivors among the 100 mice at risk being plotted against time (Topley, Greenwood & Wilson 1931). The ten upper curves were obtained in ten experiments carried out with a particular strain of *Bacterium typhi-murium*. It will be seen that this strain caused only a moderate mortality in the mice exposed to risk, though a large series of other tests had shown it to possess a relatively high virulence when injected directly into the tissues. The three lower curves were obtained in experiments carried out with a different strain of the same organism. It will be seen that this strain caused an appreciably higher epidemic mortality. The variations with each strain are considerable, but there can be no doubt as to the difference between them. The strain that failed to kill many of the mice did not fail to spread beyond those that it killed. In six of the ten experiments with this strain, the proportion of infected mice among the survivors killed on the 60th day varied from 23 to 40 %. From the survivors of the remaining epidemics initiated with this strain 100 mice were taken at random. The same number were taken from the survivors of the three more severe epidemics initiated with the other strain. These two herds were tested separately for resistance, by adding to each 25 mice infected with a third strain of *Bacterium typhi-murium*, known to produce a high epidemic mortality. As a control, three herds, each of 100 normal mice, were tested at the same time in the same way. On the 60th day 69 % of the survivors from the mild epidemics remained alive, 66 % of the survivors from the severe epidemics, and 36·7 % of the normal mice that had not previously been exposed to risk.

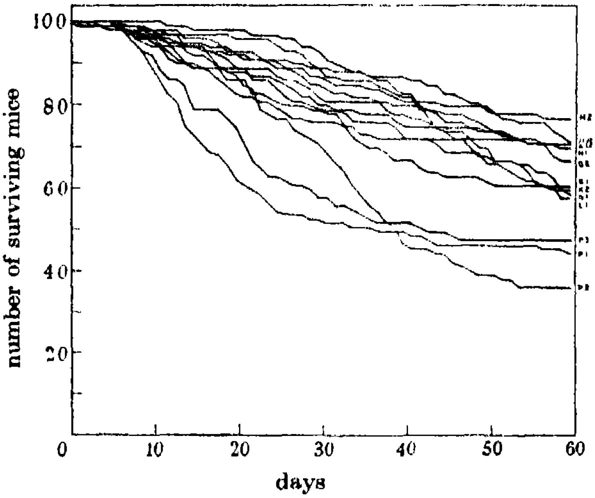


FIGURE 12

The main interest of this experiment lies in the demonstration that one strain of a bacterial parasite may, as it spreads by contact, kill more hosts than it immunizes, while another may immunize more than it kills. We have, of course, long been aware that an effective specific immunity may be conferred by mild, or latent, infections; and Dudley's prolonged and detailed studies at the Royal Naval School at Greenwich (Dudley 1923,

1926) provide a convincing demonstration of the epidemic immunization that accompanies any outbreak of diphtheria. It seems possible that infective strains, defined as those that have a relatively high power of contact spread, may be subdivided into those with high and low ratios of killing to immunizing potency.

TABLE 1

	infectivity		virulence deaths among 5 mice per dose				
strain	contact deaths	survivors infected	10^5	10^4	10^3	10^2	10
P.64	73/100	6/27	5	5	5	5	4
P.62	62/100	7/38	5	5	4	4	2
P.29	26/100	0/74	—	5	4	4	2
P.58	18/100	1/82	3	1	0	1	0
P.A.39	13/100	0/87	5	4	5	4	2

Experiments with various strains of *Pasteurella* (Greenwood *et al.* 1936) have given a still clearer illustration of the independence of virulence, as tested by the power to kill when bacteria are injected directly into the tissues, and epidemicity as tested by the numbers of mice that succumb to contact infection. Table 1 shows the results obtained with five different strains of *Pasteurella*, which are listed in the first column in descending order of epidemicity. It will be seen that strain P.64 caused the death of 73 of 100 mice by contact infection within 60 days, and infected 6 others without killing them. It was also highly virulent, 10 *Pasteurella* killing 4 of 5 mice on injection, and larger doses killing all mice receiving them. Strain P.62, within the margin of error of such experiments as these, is of the same order of epidemicity and virulence as strain P.64; but strain P.A.39 and P.29 have the same high virulence as strain P.62, but are at low epidemicity, showing very little of contact spread. Strain P.58 has the same low epidemicity as strain P.A.39, but is also of low virulence. It is clear that the biological properties, whatever they may be, that enable a bacterium to multiply within the tissues, causing the death of the host, are different, and dissociable, from those that enable it to obtain a foothold in a new host, to which it is transmitted by natural contact infection. The nature of the factors involved is at present quite obscure, and offers a fertile field of study to the bacteriologist and to the biochemist.

It may be noted that all the evidence at present available is against the view that variations in the epidemicity or virulence of the infecting parasite play a part in the fluctuations in mortality that may occur during a long-continued epidemic, or that follow changes in the closeness or continuity of contact between infected and non-infected hosts. In this respect our own experience is in accord with the views expressed by Webster and his colleagues (Webster 1923*a,b,c*, 1924*a,b,c,d*, 1925, 1926, 1930*a,b,c,d*; Webster & Burn 1926 *a*, *b*, 1927 *a*, *b*, *c*) whose independent studies at the Rockefeller Institute began soon after our own in this country. In observations lasting for more than twenty years we have only twice obtained evidence of a change of this kind occurring during experimental epidemics (Topley, Greenwood, Wilson & Newbold 1928; Greenwood *et al.* 1936). So far as our present evidence goes, it seems likely that the evolution within any parasitic species of a strain of high epidemicity is an occasional event, rather than part of a normal or periodic process.

THE EFFECT OF GENETIC DIFFERENCES IN RESISTANCE WITHIN A SINGLE HOST SPECIES

Just as different strains within a single parasitic species differ in their power of infecting, and killing, a particular species of host, so different strains, or races, within a single host species differ in their resistance to attack; and the dissociation of different infective powers that can be demonstrated in selected strains of a given parasite has an analogy in the dissociation of resistance towards different parasites, or towards different activities of the same parasite, that can be demonstrated in selected strains of host.

Several observers have been able, by selective breeding, to produce strains of laboratory animals unusually resistant, or unusually susceptible, to particular bacterial parasites (Hill 1934). In some instances, late generations of these selected strains, or mice belonging to inbred strains that have been found to be abnormally resistant, or abnormally susceptible, to a particular bacterial parasite, have been tested against other bacteria, viruses, or toxins. Thus, Gowen & Schott (1933) tested two inbred strains of mice, Silver and S., against *Bacterium typhi-murium* and against the virus of pseudorabies. S. was approximately four times as resistant as Silver to *Bacterium typhi-murium*, but Silver was approximately twice as resistant as S. to pseudorabies. Webster (1933) produced strains of mice abnormally resistant, or abnormally susceptible, to *Bacterium enteritidis* by selective breeding through several successive generations from mice that had survived infection with this organism. He then tested these selected strains against three other pathogenic bacteria, *Pasteurella*, Friedländer's bacillus, and the pneumococcus, and also against the virus of louping ill. The strain that was abnormally resistant to *Bacterium enteritidis* was also relatively resistant to the three other bacteria, but it was less resistant to the virus of louping ill than the strain that was abnormally susceptible to *Bacterium enteritidis*. Hill, Hatswell & Topley (1940) produced a strain of mice abnormally resistant to the injection of a partially purified toxin isolated from *Bacterium typhi-murium*, by selective breeding in successive generations from mice that had survived large doses of this substance. Late generations of these toxin-resistant mice were tested against living bacilli of the same bacterial species, but proved no more resistant than unselected controls. It is clear, therefore, that genetic resistance, while not specific in the same sense as the resistance depending on antigen-antibody reactions, is limited in its range, and is an expression of the effect of different factors that may be inherited independently.

The racial differences in resistance depending on these factors, in man or in animals, will affect the natural spread of epidemics by increasing their severity in some quarters of the world, and lessening it in others. They may also, in areas inhabited by a mixture of races, lead to a differential incidence or death-rate. It should, however, be emphasized that it is usually a matter of the greatest difficulty, when faced with recorded observations of this kind, to disentangle the effects of genetic factors, immunization by previous infection, and environmental influences.

The plant pathologist and the plant geneticist have gone further in this particular field, and have obtained results of the greatest interest. They have, for instance, demonstrated, in their studies on the stem rust of wheat, that the genetic resistance of selected host strains may be narrowly related to strain differences in the parasite (see Craigie 1940). A selected strain of wheat that is highly resistant to one strain of rust may be highly susceptible to another; so that the possibility that has here been opened to us of eliminating susceptible hosts by selective breeding depends on our ability to produce, by appropriate crossing, strains of wheat that are resistant to all the strains of rust by which they are likely to be attacked.

Apart from innate differences in resistance, and acquired differences in specific immunity, host resistance may be affected by a variety of other factors, by certain dietetic deficiencies, by exposure to severe climatic changes, and so on. It would take us too far afield to discuss these factors in any detail. We need only note that it is desirable to assess their relative importance by experiment, or by carefully controlled observations, before attempting to assign to them their respective roles in the natural course of events.

More complex systems

We have so far limited our attention to the simplest form of epidemiological system, in which a single species of parasite reacts with a single species of host; though each species may be divisible into biological races or types that differ significantly in their behaviour.

In turning to more complex systems, we can do no more than note analogies. The most important natural modification in our simple system is, perhaps, the intervention of an insect vector; and of all human diseases conveyed by insect vectors malaria has pride of place.

So far as prevention is concerned, our main problem is still to reduce the probability of effective contact between a person infected with the malarial parasite and a person susceptible to it. But effective contact no longer means mere propinquity. The infected person must be bitten by a mosquito of the appropriate species at the appropriate time, when parasites at the right stage of development are present in the circulating blood, and this same mosquito must then bite a susceptible, non-infected host, again at the appropriate time, when the parasite has passed through its sexual cycle in the mosquito's tissues. In the absence of mosquitoes of an appropriate kind, and in adequate concentration, the malarial parasite cannot pass with effective frequency from host to host, however closely the hosts are aggregated; indeed, the aggregation or dispersal of infected and susceptible hosts now loses its significance, except in so far as it is related to the distribution of the insect vectors, to their powers of flight, and to their feeding habits.

I have neither the knowledge nor the time to discuss the fascinating story of the prevention of malaria by mosquito control. There are, however, two points in the epidemiology of malaria that may be noted, since they bear directly on the general principles that we have considered in relation to simpler systems.

The first concerns the malarial parasite itself, and the host's reactions to it. There was a tendency until quite recent years to assume that the problem of resistance to protozoal infections in general, and to malaria in particular, differed in some fundamental way from that of resistance to bacteria and viruses. In particular, little attention was paid to the possible effect of mechanisms depending on antigen-antibody reactions. This neglect, though a natural result of difficulties in technique, was always biologically unjustified, if only because the production of specific antibodies is a general phenomenon that follows the introduction into the animal tissues of any foreign protein, or any foreign chemical complex of which a protein is a part. It is possible that this property may be shared by other large and complex molecules that have no protein component. Recent studies in malaria, particularly on monkey malaria, and on the artificial infection of human subjects as a method of cure in certain mental diseases, have shown quite clearly that there is a specific acquired immunity depending not only on the species of malarial parasite, but on strain differences within a species that are clearly analogous to the different antigenic types of bacteria and viruses. The role of antigen-antibody reactions may well be less important in protozoal than in bacterial or virus infections, but it is, perhaps, equally probable that mechanisms other than those dependent on such reactions are more important in acquired resistance to bacteria and viruses than has been commonly supposed.

The second point concerns the insect vector. Just as we have seen that different biological races within a single parasitic species, quite apart from differences in antigenic type, differ significantly in their epidemiological behaviour, and that different strains within a host species may show wide differences in genetic resistance, so we find that different strains, or races, of a single species of insect vector may differ profoundly in their efficacy as infecting agents, usually because of differing predilections for the blood of man and other animals (see Hackett 1937).

Time forbids consideration of the further complexities that may be introduced into our epidemiological systems by the intervention of alternative or reservoir hosts, or of many other factors that we have left untouched; but there is one last point that should be underlined. Whenever we seek to lessen the frequency of an infective disease, we seek, in one way or another, and usually by synchronous attack along many different lines, to reduce the probability of a pathogenic parasite gaining access to a susceptible host. We need never, by our own action, reduce the probability to zero. The biological system on which any endemic or epidemic prevalence depends is in unstable equilibrium, shifting now to the advantage of the parasite, now to that of the host. As we reduce the frequency of effective contact, we reduce the mass of infective material on which the probability of further diffusion in part depends. If we can tip the balance far enough the system itself will do the rest, and the disease will be reduced to negligible proportions, or even disappear.

In conclusion I should wish to express my indebtedness to Mr W. T. Russell, Mr W. J. Martin and Mr E. Lewis Faning for the help they have given me in obtaining certain statistical data, and in the preparation of some of the graphs.

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A contribution to the chemistry of pterins

By Frederick Gowland Hopkins, O.M., F.R.S.

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The experiments in this paper have been chiefly concerned with a derivative from pterins, the wing pigments of pierid butterflies. These pigments have become prominent of recent years, partly on account of the prolonged efforts (1925–41) of continental workers to determine their nature. The progress of these constitutional studies has quite recently become dramatic, as is made clear in the historical introduction below. Moreover, one pterin at least has been shown to occur in mammalian tissues, with probable physiological functions there. From one pterin (erythropterin) the yield of the derivative with which the present study has dealt is large. From another (xanthopterin) it arises only in special circumstances which are of considerable interest. The substance gains in interest from its mode of origin. This involves an oxidation by molecular oxygen in solutions of low pH. On the acid side of neutrality its formation proceeds throughout a wide range of pH values, but not at all on the alkaline side. The properties and behaviour of this derivative (which in some respects are exceptional) are described, and among the experimental results obtained are some which offer guidance for future extension. The ground covered has been limited, however, by difficulties at the present time in obtaining an adequate supply of material. No evidence bearing on the actual constitution of the product has yet been obtained, and the paper represents what is essentially a preliminary study. It seems justifiable to claim, however, that until the structure of this derivative is determined and the mechanism of its formation made clear, the chemistry of the pterins will remain incomplete. Another section of this paper deals briefly with the yellow products which occur when aqueous suspensions of uric acid are heated in sealed tubes at high temperature. These show properties strikingly akin to those of the pterins, and in particular yield purple derivatives in precisely those circumstances which lead to the formation from the natural pigments of the product here dealt with which they closely resemble.

1. HISTORICAL

More than 50 years ago (Hopkins 1889) I first called attention to the circumstance that a yellow pigment in the wing scales of butterflies belonging to the family Pieridae, and highly characteristic of that group of insects, is freely soluble in hot water, while preparations of the extracted pigment give a characteristic murexide reaction suggesting a relationship with uric acid. I also referred to experiments which seemed to show that it might be related to a yellow product which, as Wöhler showed in 1853, is formed when uric acid is heated with water in sealed tubes at 100–140° C. These were the first observations to be made on pigments which have now come to be known as *pterins*.

Some year later (Hopkins 1895) I was able to publish a fuller account of the yellow pigment, and I then described experiments which had convinced me that the wing scales of white pierids, e.g. of the common garden white butterfly (*Pieris brassicae*), contain uric acid itself.

These publications evoked no experimental work by others till nearly 40 years after the appearance of the first of them, and 30 years after that of the major paper. In 1925, however, Heinrich Wieland, with members of his school, began a series of researches on these substances which are remarkable for the enterprise displayed in obtaining the great numbers of insects which proved necessary for effective studies, and for the long-continued efforts made by many workers to determine the constitution of these pigments. Meanwhile, during recent years these efforts, devoted to a problem which might seem to be remote in its bearings, have apparently been justified from facts revealed in a biological field of inquiry. Thus Koschara has separated from human urine a pigment which, on what seems to be satisfactory evidence, is a pterin, closely related to and possibly identical with the xanthopterin of butterfly wings. This substance is present in mammalian tissues and may possess physiological functions, sharing for instance with the flavines an influence in tissue oxidations (Koschara, von den Siepen & Alared, 1936, 1939, 1940). Jacobson (1939) has shown that the so-called argentaffine cells of the intestinal epithelium contain a yellow pterin, while the localization of these cells agrees with that of the substance active against pernicious anaemia. Such coloured cells have been found absent in cases of that disease, and Koschara states that in such cases there is an increased excretion of uropterin. It is by no means sure, however, that the pterin itself is active in preventing clinical anaemias, though Tschesche & Wolf (1936, 1939) make the remarkable claim that the injection of 10 μg. xanthopterin causes a considerable new formation of red corpuscles in rats made anaemic by feeding on goat's milk, while progress of the anaemia is arrested by small daily intramuscular injections of the pterin as obtained from butterflies!

If such findings are ultimately confirmed and extended, these pigments have a wide interest, and the determination of their constitution is highly desirable. The solution of this problem proved to be highly elusive, however, and the history of progress towards it is in many ways remarkable. Recently it has become even dramatic. After 14 years of experimental labours (1925–39) provisional conclusions had been reached which, during the earlier months of 1940, were shown to be illusory. Interesting syntheses now seem conclusively to have proved, in the case at least of two of the more important pterins, that they have constitutions differing from, and much less complex than, those previously assumed for them (p. 362).

In a recent return to this field, on limited lines, after 45 years, I began experiments which I hoped might throw some light on the constitutional question. This possibility has perhaps lost its interest to-day when that question seems settled. The results I have to record may, however, be found to have some interest of their own, though many of the suggestions they carry could not be followed up from inability, during recent years, to obtain the requisite material. They may provide guidance for future experimental work. They relate in particular to a derivative from the coloured pterins that received some attention in my paper of 1895, but which was not then isolated. As a product it has proved to be of quantitative importance, and it arises from its precursors on lines which seem to add to its interest.

Another section of this paper describes some extension of the evidence, which was also briefly dealt with in the 1895 publication, showing that certain yellow products obtained when uric acid is heated with water in sealed tubes at high temperatures have apparent affinities with the yellow pterins.

Even the briefest reference to the earlier among the many German publications would be impossible here, but enough may be said to make the present position clear.

The chief studies have dealt with the white pterin from *Pieris brassicae* and similar species, and with the light yellow pigment which characterizes such insects as that familiar in England as brimstone butterfly (*Gonepteryx rhamni*). The former is known as leucopterin and the latter as xanthopterin. One other coloured pterin of importance has also been studied, but, so far, less effectively. This is the *erythropterin* of Schöpf & Becker (1933). It will be found to be important in the experiments described in the next section.

Leucopterin as a crystalline substance easily individualized, and for a long time yielded the clearest results. It was this substance that I formerly believed to be uric acid. Its constitution is notably different from, though not so remote from, that of the acid as was thought till quite recently.*

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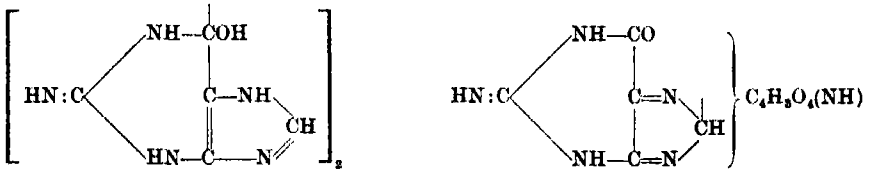
* The close resemblance between leucopterin and uric acid in respect of their general physical characters has been more than once emphasized by the German authors (Wieland & Schöpf 1926; Schöpf & Becker 1936). It is interesting to learn that Tartter (1940), working on a very large scale, has isolated uric acid itself from the wings of white pierids.

The study of xanthopterin offered greater difficulty, but in 1939 it was shown (Wieland & Purrmann 1939) to possess the same fundamental carbon structure as leucopterin. This circumstance, though now based on somewhat modified evidence, remains certain. In the paper just mentioned are found the provisional structural formulae for the two pterins in question, which represented the views of the Wieland School at that date. It will be seen that of the nineteen C atoms which they were believed to contain, fifteen were in each case included in three purin residues, all identical in the molecule of leucopterin, while in that of xanthopterin one differed in detail from the others. The former contrasted with the latter in containing oxygen at position 8 of the purin structures. This was still the view of the Wieland school when my own experiments had to cease.

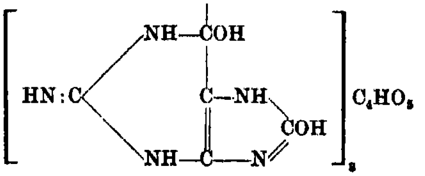
The chief evidence for the striking alteration of outlook which occurred last year will be found in three papers, the first by Wieland & Purrmann (1940) on 'Leucopterin and xanthopterin', and the second by Purrmann (1940*a*), 'Synthesis of leucopterin and the nature of guanopterin', and the third by Purrmann (1940*b*) on the 'Synthesis of xanthopterin'.

It would seem to be now proved that the leucopterin molecule, like uric acid and other typical purins, contains one pyrimidin ring only, but in association not with the five-membered iminazol ring of the purins but with a six-membered ring containing four carbon atoms. Nevertheless, it will be seen that its relationship with uric acid is not very remote. Xanthopterin is 8-desoxy-leucopterin and can be easily oxidized to the white pterin.

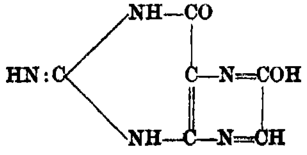
It is remarkable that the new formulae represent compounds with molecular weights only one-third of those involved in the earlier conceptions.



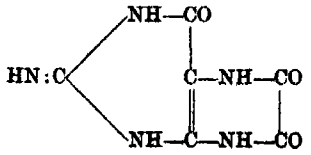
Xanthopterin (1939)



Leucopterin (1939)



Xanthopterin (1940)



Leucopterin (1940)

2. RHODOPTERIN (FORMERLY 'LEPIDOPORPHYRIN')

A red-purple derivative from yellow pterines

In 1895 I called attention to the fact that yellow pigments from the wings of pierids when heated on the water-bath with 20% sulphuric acid yield a purple derivative with high tinctorial power. This was true of the wing pigments from all the species then studied. The product, whatever its source, shows in acid solution two characteristic absorption bands identical in every case (infra). At that time I called it lepidoporphyrin, a name unsuitable to-day when the term porphyrin carries such definite and quite different implications. As present evidence suggests that as a derivative it still retains the main structure of a pterin, I propose in the present paper to refer to it as *rhodopterin*. The German authors paid little or no attention to this derivative; chiefly, I think, because their methods did not reveal its quantitative importance. My recent experiments have shown, however, that it is obtained in relatively large yield, and is of special interest in that it arises from the pterins, or from certain among them, as the result of quite mild treatment. This involves heating with N/10 sulphuric acid at water-bath temperature with an air stream passing through the solution kept at constant volume by the use of a vertical condenser. The formation of the product involves an oxidation by molecular oxygen, as no trace is formed when the conditions are made strictly anaerobic by first passing a stream of nitrogen through the apparatus and fluid. Decinormal acid is a convenient strength to employ. The original pterins are soluble in this when hot, while the rhodopterin is completely insoluble, separating immediately it is formed; the production is relatively rapid. The oxidation proceeds, however, though more slowly, with much lower concentrations of acid. The effect of the pH of the solution is indeed noteworthy. At pH 7·6 for instance rhodopterin formation does not occur at all, while on the acid side of neutrality it still fails at pH 6, which is about that of simple aqueous solutions of the pterins. At pH 4·6, however, the production of the purple derivative becomes evident. Increase of alkalinity has no effect until it becomes large, when the pterin is otherwise decomposed, whereas with increase of acidity at least up to that of 20% sulphuric acid by volume its formation proceeds normally. I have failed to learn of any recorded case of oxidation by atmospheric oxygen to which such facts as these apply. Indeed, even cases in which acidity of solution favours such oxidation rather than alkalinity seem to be rare.

Rhodopterin can be obtained crystalline without difficulty in the form of microscopic needles almost black in colour. The conditions for success are described in the Experimental Section.

It is not difficult to obtain satisfactory estimations of the yield of the product even when working on a small scale. Its complete separation from solutions in which it has been formed is easily secured at the centrifuge, and as it is absolutely insoluble in the weak acid and in boiling water, it can be thoroughly washed free from soluble products without loss.

The following percentage yields obtained at an early stage in the investigations are worth reporting in illustration of the wide distribution among pierids of the precursor or precursors of the purple product, and in showing that as a breakdown product it is quantitatively of importance. The actual percentage figures in the table, though accurate as estimations, and of value in their comparison of different species, are otherwise of limited significance. They were obtained from hot-water extracts of the prepared wings (see Experimental Section), though these contain more than one individual pterin, while in most cases mixed species had to be employed.

	species of origin and numbers employed, with character of wing colour	total of mixed pigments from wings mg.	yield of purple derivative %
1.	*Terias proterpia* (121). Deep brownish orange	0·073	20·0
2.	*Terias hecabe, T. drona, T. venata* (530). Small species of pale lemon yellow colour	0·334	17·4
3.	*T. nicippe* and *reticulata* (128). Pale lemon yellow	0·127	12·2
4.	*Ixias pyrene* and *marianne* with *Herpaenia emma* and *judith* (220 in all). Deep orange yellow	0·173	33·0
5.	*Colias crocea* [=*edusa*] (218). Full orange yellow	0·175	18·0
6.	*Colias fieldii* (1118). Full orange yellow	1·121	18·5
7.	mixed unidentified species (100); all pale lemon yellow	0·52	19·0

It should be said here that in no circumstances does leucopterin yield this purple product.

I may remark that most of the more recent experiments to be described were carried out on the species *Colias crocea* [=*edusa*] and *C. fieldii*, a closely related species. Of these I have dealt with about 3000, while of any other species (mostly foreign) it has proved impossible at this time to acquire more than some 100 individuals at most. Thus in order to obtain reasonable yields of rhodopterin, I have been often compelled, as mentioned above, to employ mixed species.*

A laborious research by Schöpf & Becker (1936), in which they showed that the wings of pierids commonly, if not always, contain a mixture of pterins, made it appear necessary to discriminate among them as precursors of rhodopterin. These authors dealt with five different species, choosing cases in which the wings are orange yellow or reddish in colour, rather than the lemon yellow of species in which xanthopterin is the most prominent pigment. In the case of four of these species they worked with some thousands of individuals. They showed that in these insects a pterin quite distinct from xanthopterin, though in association with the latter, is responsible for the deeper coloration. For this they proposed the name *erythropterin*. Other constituents, still, apparently, to be classed as pterins were distinguished, but these were very small in amount. They do not call for attention here.

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* The German authors have been remarkably successful in obtaining adequate supplies for their extensive studies. At a relatively early stage (Wieland, Metzger, Schöpf & Bülow 1933) some quarter of a million of *Pieris brassicae* had become available as a source of leucopterin. It is of interest to learn that the Bavarian Minister of Education had instructed schoolmasters to supervise the catching of these insects by their scholars during the summer of 1934–5. For their chief study of xanthopterin Schöpf & Kottler (1939) employed 16,000 *Catopsilia rurina*. Tartter (1940) recently extracted the wings of 850,000 white pierids.

One realizes from a study of Schöpf and Becker's work how exceptional are the difficulties in the task of separating and purifying these substances, due, perhaps, in part at least, to their possession of a common basic structure. No actual estimations of the amount of the individual substances identified in the wings were attempted. Erythropterin undoubtedly makes a chief contribution to the coloration of the more deeply coloured pierids.

In an endeavour to decide on the origin of rhodopterin only erythropterin and xanthopterin need particular consideration. The former when obtained relatively pure is red in colour in sharp contrast with the lemon yellow of xanthopterin. In N/2 acetic acid its fluorescence is violet while that of xanthopterin is yellow. Its barium salt, unlike that of the latter, is very insoluble in hot water. It is less basic than the yellow pigment so that its isoelectric point is at a pH much more on the acid side. This last circumstance was largely responsible for whatever success was reached by Schöpf & Becker in their endeavour to isolate and purify this red wing pigment. In brief, the method used by them involved the addition of HCl to solutions of the mixed pigments in ammonia, the addition being made in amounts determined by preliminary experiments. The precipitate thus obtained is reddish in colour. It was by repeated resolution in ammonia and reprecipitation by the acid that these authors obtained their purest product. It was not claimed however that it was successfully individualized on these lines. The elementary analyses of different preparations gave varying results, and the authors were content to suggest for erythropterin the following approximate formula:

$$C_{19}.H_{17-18}.N_{11-13}.O_{8-10}.$$

The uncertainty is unfortunate in connexion with what follows, but doubtless it is a pigment quite distinct from xanthopterin. It apparently contains notably less nitrogen, but nevertheless there is good reason to believe that the fundamental carbon structure is the same in both.

It became necessary to decide whether both of these pterins are precursors of rhodopterin. That a precursor contributes almost universally to the coloration of this family of butterflies is beyond question. Out of some thirty species displaying from pale yellow to red orange wing colours when tested qualitatively on a few individuals, not one failed to yield some at least of the purple substance when the whole pigments of the wings were employed. Nevertheless, the intensity of the purple colour obtained in the test from a given amount of material doubtless varies from case to case. In general, but not quite consistently, the amount is greater from species of orange yellow or reddish orange colour than from those which, like *Gonepteryx rhamni*, are coloured lemon yellow. It seemed possible therefore that erythropterin would prove to be the main precursor, xanthopterin not yielding it or yielding it in smaller amount. An attempt was made to reach a decision on this matter.

Schöpf & Becker's method for the concentration of erythropterin involves much loss of material which makes its application difficult when working on a scale so much smaller than theirs. An endeavour was made to discover a more economical means for the separation of erythropterin and xanthopterin, but without satisfactory results, so the original method was applied, perforce on a small scale. It may be mentioned, however, that this endeavour revealed an interesting property of the coloured pterins, namely, their ready solubility in warm ethylene glycol. At 100° C strong solutions can be obtained from which a considerable proportion separates on cooling. The solubility at ordinary temperatures is considerable however. The glycol can be satisfactorily used to remove the pigments from the wings themselves if these are first wetted with a trace of water and heated with the solvent on the water-bath. The different pterins show no selective solubility however. On adding to glycol solutions five or six times their bulk of acetone, the pigments are precipitated, though the separation is slow. It is still, I think, possible that a separation might be obtained on these lines. Erythropterin seems to be more readily thrown out by acetone.

In one experiment the material employed was from 100 individuals of mixed species, all dark orange yellow in colour. They were chiefly *Euremia proterpia* and *Ixias pyrene*. The total pigment extract, first evaporated in vacuo, was dissolved in ammonia and HCl added until the solution coloured a congo red paper blue. The resulting precipitate, reddish in colour, was centrifuged off, redissolved in ammonia and again precipitated by a slight excess of HCl. It was washed at the centrifuge and dried. It weighed 41 mg. and yielded 13·6 mg. of crystalline rhodopterin (33·17 %). The centrifugates from the HCl precipitates were lemon yellow in colour. A crude xanthopterin fraction (following Schöpf & Becker) was thrown out from these by the addition of sodium acetate. This precipitate when treated on the usual lines yielded only 4 % of the purple product.

In a second experiment the pigments from 250 *Colias edusa* were employed. The treatment was similar except that the mixed pigments were first thrown out of their ammoniacal solution by acetic acid before the HCl fractionation was applied to the precipitate. The resolution in ammonia and reprecipitation with HCl was thrice repeated. The final fraction so obtained, full red in colour, weighed 55·8 mg. and yielded 17 mg. purple (31 %). The xanthopterin fraction obtained as in the previous experiment again gave a small yield, only 3 %. In both cases, however, this fraction contained large amounts of ash. The appreciable solubility of xanthopterin in cold water makes it impossible when working on a small scale to wash such precipitates free from sodium salts.

While the above results seem adequate to show the erythropterin may well be the chief source of the purple substance, its preparations, as used, undoubtedly contained some xanthopterin, while those of the latter were clearly far from pure.

It became therefore desirable to decide whether or not pure xanthopterin yields the purple product even if in relatively small amount. For this it was necessary first to prepare the crystalline barium salt, as it seems that only by means of this can purity of a preparation be assured. The salt was first described by Schöpf & Wieland in 1926; but it is characteristic of the remarkable difficulties met with in this field of research that, apparently, unless somewhat rigorous conditions are exactly reached, attempts to get a well-crystallized product may fail. In successive papers (Schöpf & Becker 1933, 1936; Schöpf & Kottler 1939) difficulties and occasional failures are reported, and only in the latest of these papers was complete confidence expressed in the method then described. According to the Darmstadt investigators, when, as in this method, the salt is prepared by adding a solution of the pigment in ammonia to baryta solution while the latter is boiling (a crystalline product being then more certainly obtained), the xanthopterin while in association with the barium apparently contains the elements of a molecule of water not present when the preparation has involved the use of cold baryta solution. On the other hand, they claim that when the pigment is liberated from this compound it again shows the same percentage composition as the original native pterin. Consultation of the relevant papers suggests that while the published analyses seem to give good evidence for the constitution claimed for the barium salt, the evidence for that of the liberated pigment is inadequate. How far the establishment of the simpler constitution for xanthopterin bears on such distinctions is not yet clear.

During the present study, while some failures in the preparation of the barium compound satisfactorily crystalline were experienced, successful preparations were made from the pigments of *Gonepteryx rhamni* and of *Catopsilia rurina*. In experiments with these a notable circumstance came to light, any explanation of which, when available, may bear on constitutional questions. It became clear that the barium compound itself and, no less, the pigment liberated from it, yield the purple product with very much greater ease and rapidity than does any preparation of xanthopterin which has not first suffered conversion into the barium compound. These statements are based on the following observations.

For liberation of the pigment the German authors dissolved the barium salt in hydrochloric acid and threw out the xanthopterin by adding a sufficiency of saturated sodium acetate solution. As, however, it was the intention in my experiments to subject the pigment after liberation from the salt to the usual treatment for the production of the purple derivative, heating, namely, with N/10 H_2SO_4, it seemed that it should prove convenient first to remove the barium by treating the crystals with an amount of the acid just in excess of what would correspond with the barium present (41 % of their weight). It was assumed that the $BaSO_4$ could then be filtered off and the filtrate straightway adjusted to decinormal strength of acid and treated as usual for the production of rhodopterin.

In one experiment this treatment was applied to a crystalline preparation from the pigments of *Catopsilia rurina*, the species employed by Schöpf & Becker in their large-scale study of xanthopterin. On treating the crystals with sulphuric acid slightly in excess of the barium equivalent with very brief warming on the water-bath, it was seen that the $BaSO_4$ had strongly adsorbed the yellow pigment, and before it could be centrifuged off the precipitate began to darken from formation of the purple product. Some unaltered pigment could still be extracted with boiling water however, and the hot yellow extract was adjusted to the requisite acidity and duly warmed and aerated on the water-bath. In 3 min. the separation of the purple product could be easily seen, and the conversion was complete in less than 15 min. The yield calculated from the amount of the original pterin in the barium salt was rather more than 10 %. Much, however, of the rapidly formed product was lost on the barium precipitate.

In another experiment on nearly similar lines with a crystalline salt from the pigments of *Gonepteryx rhamni*, precisely similar behaviour was displayed. It then seemed desirable to observe the behaviour of the xanthopterin when liberated from the salt by the method of Schöpf & Becker. A crystalline preparation from *Catopsilia rurina* was dissolved in HCl, the pigment precipitated by the addition of sodium acetate and, after washing, dissolved in N/10 sulphuric acid and the solution heated as usual. Again the separation of the purple derivative began within a few minutes, and the conversion was rapidly completed. In a final experiment the wings of a number of mixed species, comprising chiefly various species of the genus *Euremia*, were employed for the preparation of the barium compound. In this case it remained obstinately amorphous at each stage of treatment. Nevertheless, the xanthopterin, when liberated as in the last case, yielded the purple product with similar rapidity. It was further observed that when a weak solution of this free pterin in N/10 acid was allowed to stand overnight at room temperature, conversion became complete.

In contrast with this rapid production of the derivative from barium preparations is its much slower formation from the native pterins, even in the case of erythropterin with its high yield. In the case of fractions such as those described above, in which erythropterin was concentrated by Schöpf's method, heating with aeration had to be continued for 40 min. before the formation of the product becomes obvious, and as much as 2 hr. before the process is complete. The question still arises: Does the native form of xanthopterin yield rhodopterin at all? No preparation that I have been able to make without use of the barium salt has failed to yield some, but, though always pale yellow in appearance, one could not be sure that they contained no erythropterin. It may be said that most probably 'classical' xanthopterin, that from the species employed by Wieland and by Schöpf when in its pure native form yields none.

Whether as the effect of boiling with baryta solution which is involved in Schöpf's latest method of preparing its barium salt (used in the above experiments) or for some other reason, it would seem that during the process of preparation the xanthopterin molecule undergoes a change by which in one direction at least its stability is lessened.

As stated earlier in this section, the German authors (up to 1939) had not studied any purple derivative from the pterins. In a recent paper by Wieland & Purrmann (1940), however, there is reference to a red-purple crystallized product appearing 'during the catalytic dehydrogenation of xanthopterin'. This apparently applied particularly to the barium compound. The yield was very small, and its occurrence was attributed to the presence of an impurity. The number of the *Annalen* containing this paper is not available in Cambridge or London, and the reference to this product in the *American Chemical Abstracts* is too brief to allow of a decision whether it was rhodopterin or not.

In parenthesis it may be said that it is at least possible that lemon yellow isomers of xanthopterin exist in the wings of certain species of pierids. Schöpf & Becker (1936) obtained from *Catopsilia argentea* a pigment which they called 'acid xanthopterin' which differed in certain respects from the typical form of the native pigment. While, in general, species of which the mixed pterins give relatively high yields of rhodopterin have the orange yellow or reddish colour which betokens a dominance of erythropterin, the rule seems not without exceptions. It will be seen from Table 1 that species of the genus *Terias* (item 2), though certainly pale yellow in colour, gave a good yield, and the same was true of certain mixed species (item 7). If such isomers exist, they certainly add to the complexity of chemical studies, but they may prove to be of biological interest. It may be, as is said to be true in the case of plant pigments, that a particular biological end (even the production of a particular colour) may in different species be reached on somewhat different chemical lines.

General properties of rhodopterin

The substance has proved to be insoluble in all of a large number of organic solvents tried singly or in mixtures. It is completely insoluble in boiling water. Although 1 mg. dissolved in a litre of 20 % sulphuric acid imparts a considerable colour to the solution, no trace of colour is yielded to hot water. In ammonia it is slightly soluble without undergoing decomposition, while in caustic alkalies it dissolves freely but decomposes, the purple solution gradually turning brown. In strong sulphuric acid it dissolves without decomposition, but is insoluble in the acid at concentrations below 4 %. In 5 % acid a trace is soluble at 100° C; in 15 % a small amount dissolves cold, in 20 % enough to give a strong colour to the solution. In HCl it is less readily soluble than in H_2SO_4. A character worthy of mention is its ability, when freshly prepared in its amorphous form, to assume a high grade of dispersion. If it be thoroughly ground up with various solvents in which it is actually insoluble, suspensions are obtained which it is difficult to distinguish from true solutions, though on standing for some time or more immediately at the centrifuge, complete separation occurs.

The substance has no definite melting-point.

Dissolved in strong sulphuric acid, or in such strengths of acid as are capable of dissolving it, rhodopterin shows, as I stated in my 1895 paper, two well-defined absorption bands in the visible spectrum with centres at λ 548 and λ 504. These have proved to be identical in every preparation whatever its origin.

Elementary analyses

It cannot be claimed that the data obtained for the percentage composition of the product are satisfactory. The continental workers early stated that the native pterins apparently did not yield satisfactory nitrogen figures by the Dumas method and employed a modification of Kjeldahl's method. This, however, could not be applied to rhodopterin. Moreover, in accepting the new formulae for xanthopterin and leucopterin it had to be admitted that the elementary analyses which had seemed to support the earlier assumption must have been incorrect. If it be really the case that pterins present some inherent difficulty in the application of orthodox analytical methods, it is impossible to say whether this would be transmitted to such a derivative as the purple product.

The following data were all obtained from crystalline preparations, but the substance offered the disadvantage that it could not be recrystallized.

The preparations used were derived chiefly from the pterins of *Colias edusa* and *C. fieldii*, though the supplies of these were diverse in origin. No. 7 was from the pigments of mixed species of the genus *Terias*; no. 3 gives a repeat estimation of nitrogen on the same material as no. 2; no. 8 was made on a product derived from a fraction in which erythropterin had been concentrated by the method of Schöpf & Becker.

	C	H	N	O
1	39·19	3·29	32·9	24·62
2	39·03	3·52	(35·7)	—
3	—	—	33·2	—
4	37·93	3·16	33·6	25·31
5	38·18	3·50	33·7	24·62
6	—	—	33·2	—
7	39·39	3·42	—	—
8	38·12	3·61	32·0	26·27
Average	38·64	3·42	33·1	25·20
$C_{19}H_{20}N_{14}O_9$	38·77	3·40	33·33	24·50
$C_4H_4N_3O_2$	38·09	3·20	33·34	25·18

It will be seen that there is considerable scatter among the figures for C and H, though those for N are consistent. These data were obtained when the structure of both leucopterin and xanthopterin was still supposed to contain nineteen C atoms, and the fact that the average of the above results agreed well with a nineteen C formula supplied grounds for a belief in its reliability. The consequent assumption that the carbon structure of the original pterins remained intact in rhodopterin seemed to be confirmed by the fact that during the oxidation which produces it no CO_2 is evolved.

On one occasion 70 mg. and on another 85 mg. of the mixed pigments from *Colias edusa* were oxidized on the water-bath in the usual way (in N/10 H_2SO_4), the apparatus being so arranged that CO_2-free air was bubbled through the solution and then passed through an exit tube into baryta solution. This remained clear throughout the course of the oxidation. When the fluid in the flask was subsequently made alkaline and distilled, the application of van Slyke's method to the distillate showed that no ammonia had been formed.

At this stage some discussion of relations between the purple product and the original pterins seemed possible, but with the arrival of the complete change of view concerning the nature of the latter, this has become difficult. Meanwhile it had been found that preparations such as those analysed, though the crystals were well formed and, on a cursory examination, free from admixture; when closely examined under a high power of the microscope showed in very small amounts the presence of minute yellow granules for which no discriminating solvent could be found. It was further found, however, that when a suspension of a preparation is thoroughly centrifuged (in small tapering tubes) this impurity was only found in the lowest layers of the precipitate. With care upper portions could be removed in which the crystals appeared free from all admixture.

Nearly a year later than when the first figures were supplied, two such preparations were analysed:*

	C	H	N
A	38·50	3·40	31·40
A, duplicate	38·55	3·71	31·75
B	38·13	3·59	31·66

It will be seen that the C and H figures agree sufficiently well with the average of the earlier analyses, but those for N are 2 % lower. This might of course be attributed to the removal of the impurity, though the amount of this seemed in general too small to produce the effect. A rejected preparation happened to be available however, of which the appearance had suggested that it contained an exceptional amount of the yellow substance. This was sent for analysis with the above two apparently pure products, in the hope of learning more definitely in what direction the results would be affected. It gave the following figures:

C, 38·00; H, 3·60; N, 31·66.

The figures depart relatively little from those of the two supposedly pure products. This result was disconcerting, and it must be admitted that the percentage composition of rhodopterin is yet uncertain.

It is hoped that at some future time a study of decomposition products may throw light on its nature. One method of obtaining such would seem to be of promise.

Oxidation by perhydrol

Its properties made it difficult to decide on a process likely to yield breakdown products or other derivatives of rhodopterin. It was found to be susceptible however, when in ammonia solution, to oxidation by hydrogen peroxide, yielding colourless soluble products. Since no more than some 150 mg. could be made available for the purpose, a study of the results of this oxidation in the present research could at most be of a preliminary kind. Sufficient experience was gained, however, to suggest that with the use of enough material its application should throw light on the constitution of the product. As already stated, the substance is only slightly soluble in ammonia. If, however, the oxidation is carried out with frequent shaking of the fluid, a quantity of ammonia much smaller than that required for complete solution suffices. As the oxidation proceeds, undissolved material continuously takes the place of that already oxidized, and the process goes to completion. The perhydrol used in the experiments was a special preparation leaving no trace of residue when evaporated. (When quantities similar to those employed in the experiments were added to ammonia and the mixture evaporated on the water-bath, the very slight residue contained traces of ammonium nitrite and apparently minute amounts of hydroxylamine nitrate.)

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* In the same laboratory, that of Drs Weiler and Strauss at Oxford.

The following procedure has given satisfactory results. The rhodopterin is suspended in 2 N ammonia (25 c.c. for each 50 mg.) and the flask placed on a water-bath. The perhydrol is added slowly little by little with vigorous shaking after each addition. When all but a small part of the substance has gone into solution and the fluid has acquired a light brown colour, the flask is left on the bath without shaking for about 15 min. so that the temperature is allowed to rise till near boiling. The fluid may then have become colourless. If not, further small additions of perhydrol are made and shaking continued till all colour has disappeared. It is finally heated for a short time longer until there is no obvious evolution of oxygen on shaking. The solution is then evaporated in vacuo. The white residue should give no peroxide reaction. The whole is soluble in water and the solution is acid. It is apparently insoluble in organic solvents. Efforts were made to separate individual products from such residues, but as they were finally without complete success owing to the small amounts of material available, the present paper must not be lengthened by their description. A strong impression was gained however that with a reasonable supply of rhodopterin for oxidation, satisfactory isolations might well prove relatively easy.

Discussion

At the time when some preliminary observations had suggested that the purple product would prove worthy of study (1939) certain dealers were still in touch with agents or representatives abroad, and I was assured that there might be no difficulty in obtaining coloured pierids in quite large numbers. Soon afterwards, however, contacts of the kind were completely interrupted, and I had to seek for supplies in this country. Many collectors have helped me, but it will be understood that the number of a given species which could be spared from private collections, and even from those in museums, could not fail to be relatively very small.

Ultimately it became clear that the study would have to be on more limited lines than I had hoped might be the case.

It is unfortunate that some experimental results of promise could not be followed up, the oxidation by perhydrol for instance. Yet it has seemed that the results obtained are worthy of publication, as they may give guidance for extended experiments should these become at any time possible. The following aspects of the present position seem worthy of consideration. First, the relations of the purple product with the *erythropterin* of Schöpf & Becker. Erythropterin is a pterin which probably plays as large a part in the coloration of the pieridae as does xanthopterin, and it is unfortunate that hitherto it has not been obtained pure.* It is sure that further studies of erythropterin and rhodopterin would be mutually helpful. Again, if the observed rapid and relatively large yield of the product by xanthopterin, when it has first been converted into its barium salt, is confirmed by others, the circumstance certainly offers a constitutional problem of interest. One would like to know in proof of their identity if synthetic xanthopterin behaves the same in similar circumstances. It is of interest to remember that leucopterin fails to yield the product in any circumstances. If the now accepted formulae for xanthopterin and leucopterin be compared, it would seem that the existence of oxygen at position 8 in the latter must in some way prevent the particular oxidation involved in the formation of rhodopterin. In any case it seems fair to claim that until the constitution of this derivative becomes known, knowledge of the chemistry of the pterins will remain incomplete.

The fact that during the oxidations which produce it no carbon is removed as CO_2 suggests that it retains the essentials of a pterin structure, but if so it is likely that the complexity of its molecules is increased by an association between two or more pterin groups.

3. DERIVATIVES FROM URIC ACID SHOWING RELATIONSHIPS WITH NATURAL PTERINS

So far back as 1853 Wöhler showed that in the course of heating uric acid in sealed tubes at high temperatures a yellow product is formed, and about the same time Hlasiwetz erroneously claimed that it was identical with the 'Mycomelic acid' which had been previously described by Liebig. In my earlier papers I called attention to the close resemblances displayed by this product (or products) and the natural yellow pterins. During the later stages of the present research the evidence for this has been confirmed and somewhat extended, but only on limited lines. To obtain a maximal yield of the yellow products the amount of heating to which the sealed tubes are submitted has to be defined with respect to both time and temperature. What appears to be a maximum is reached by heating for 6 hr. at 195–200° C. Needless to say hydrolytic products in relatively large amount are present in the heated tubes, ammonium urate predominating. By fractionation of an aqueous solution, however, the yellow products can be greatly concentrated. The following may be said of fractions which have been so concentrated, though with no pretence to even approximate purity. Alike in the solid form or in solution they closely resemble in appearance pterin preparations; almost exactly, for instance, one of the mixed pigments of *Colias edusa*. In aqueous solutions the fluorescence is similar in both, and shows the same variations in intensity with changes of dilution and pH. The general solubilities are the same and include the special case of ethylene glycol which freely dissolves both, though both proved equally insoluble in various other organic solvents tested.

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* If any fresh work has been done on this pterin during the present year, the results have not yet appeared in any form available in this country.

Among these and other similarities one is outstanding because so characteristic and special. The products from uric acid yield purple derivatives in precisely those circumstances which lead to the production of rhodopterin from pterins. If fractions from the tubes such as those referred to above are heated on the water-bath with N/10 sulphuric acid, no change is observed if anaerobic conditions are maintained. So soon, however, as an air stream is passed through the solution the formation of purple derivatives begins, though somewhat more slowly than is the case with the pterins. Heating with aeration for 5 or 6 hr. may prove necessary for complete conversion. These products differ from rhodopterin in being completely insoluble in cold 20 % H_2SO_4, though appreciably soluble in it when hot. It is therefore convenient, when the tube contents are being fractionated and individual fractions tested, to use the stronger acid since the solution gradually gains in colour as the process proceeds, and the relative concentration of the precursors in various fractions can be assessed straightway. Complete separation occurs when the solution becomes cold. In my earlier studies I found that the absorption bands in the visible spectrum shown in sulphuric acid solution were commonly identical in the uric acid derivative and in rhodopterin, namely, the two with centres at λ 548 and λ 504. I observed, however, that a third band at λ 600 appeared in some preparations. Much of the effort spent in this further investigation has been concerned with the significance of this extra band. There is an aspect of the behaviour of the uric acid derivatives at the spectroscope which must be borne in mind. In concentrated sulphuric acid solutions, unlike those of rhodopterin, no bands are clearly visible whatever the concentration of the solute. In acid of 50 % by volume some preparations show all three bands plainly. Yet in these cases on dilution of the acid to 25 % the λ 600 band entirely disappears and with it a characteristic blue shade in the solution which is always associated with its presence. On its disappearance the colour becomes that of a solution of rhodopterin and the two bands so characteristic of this alone remain. This effect of varying the strength of acid holds when the concentration of the solute is kept constant. In other preparations the central band at λ 543 may appear to be the strongest of the three (infra). Clearly the spectrum should always be first observed in solutions containing about 50 % acid. It is sure, however, that preparations may be obtained which even then show no band at 600, but only the two which are identical with those of the products from pterins. My colleague, Dr R. Hill, who has kindly made a spectroscopic examination of very numerous preparations, is of the opinion that the variations are explained by the existence of two distinct forms of purple substance, the one giving with exactness the two bands of the pterin derivatives and these alone; the other responsible for the λ 600 band together with another corresponding nearly, but not quite, with the more redward band of the former. In weak solutions of the mixed products this middle band may therefore appear as the strongest and is somewhat wider than when seen in the rhodopterin spectrum. This of course suggests that two somewhat different forms of the yellow precursor are produced from uric acid. It would seem that there is also a third which fails to yield a purple derivative. This is also true, however, of the mixed pigments from certain species of pierids. The significant fact remains that with the sealed tube technique a product can be obtained from uric acid which, apart from other similarities, yields in similar circumstances a derivative which must contain in its structure a chromophore group identical with one in the product from yellow pterins. It is scarcely possible that such a product from uric acid can be identical with rhodopterin, but it would seem that there must be close relations between them. This is perhaps more easy to understand with the new formula of the pterins than with the old.

There is, moreover, another aspect of the behaviour of the purple derivatives from the sealed tube products which emphasizes their resemblance to rhodopterin. Like it they are oxidized in ammoniacal solution by perhydrol, yielding colourless acid products.

4. EXPERIMENTAL

The procedure employed in all preparations of the coloured pterins was the following. The wings are first extracted with hot ethyl alcohol (97 %), then thoroughly extracted with ether and afterwards with cold water in which they remain overnight. The last extracts from some species a green 'interlaminar' pigment (Hopkins 1895), and sometimes from others a small amount of yellow which does not seem to come from the scales. The wings are then extracted with hot water in the following way. They are first just moistened with alcohol to secure 'wetting', and in a beaker are then covered with hot water in quantity just sufficient to permit of free stirring to break down coherent masses. The water is then raised to boiling and brisk stirring continued. It is easy to see when the coloured areas on the wings have become pigment-free. If this does not occur with the amount of water first used, more should be added (hot) in successive small amounts till it becomes the case. Extraction with the minimum adequate amount of fluid is thus secured. The extract is then filtered hot. A considerable amount of the pigment separates when the filtrate cools. When, however, no attempt of fractionation at this stage is intended the whole extract is evaporated in vacuo. Before any further procedure is applied to the product it is dissolved in a minimal amount of ammonia and reprecipitated with acetic acid. This involves a small loss of the more soluble pigments, but the product is thus freed from the mineral impurity which is sometimes appreciable in spite of the preliminary treatment suffered by the wings.

Preparation of rhodopterin

The general nature of the quite simple method used for the preparation will have been understood from statements in the last section, but a few details may be added here. The wing pterins are heated in a flask on the water-bath with N/10 H_2SO_4. A glass tube passing through a vertical condenser carries an air stream through the solution. Actual boiling should be avoided, as an adequate concentration of dissolved oxygen is then not maintained. The fluid in a flask with about half its bulk exposed directly to the steam from the bath and receiving the returned water from a vertical condenser maintains a temperature of about 85° C at which the process proceeds satisfactorily. It has been earlier emphasized that the product separates from the hot decinormal acid directly it is formed, and as the process proceeds the solution is seen to grow cloudy from its separation. After about 2 hr. the conversion is complete, and on standing the product settles out so completely that a great part of the mother liquor can be poured off without loss. It is finally separated at the centrifuge and afterwards boiled several times with water in order to remove traces of soluble impurities. So obtained the product is commonly amorphous. Yet if a mere trace of previously prepared crystalline material has been added to the contents of the flask before heating begins, the whole yield has never failed to be crystalline.

It is desirable to obtain a small stock of crystals for sowing purposes by proceeding on quite a small scale as follows. The desiderata are low concentrations of the pterin employed and (apparently) a slow supply of oxygen at the start. If, say, 10 mg. of the mixed pigments of *Colias edusa* are heated on the water-bath in 40 ml. of the decinormal acid *without an air* stream, there is enough dissolved oxygen in the fluid to complete the oxidation. The heating should be continued for about 2 hr., when after cooling the precipitate is centrifuged off. The amount is of course very small, but if it be preserved in a test-tube under 2 or 3 ml. of distilled water, it will serve for sowing in a number of preparations on a much larger scale with the use of an air stream.

When more convenient it is easy to get the same result by using direct the hot-water extract from a small number of insects choosing a deeply coloured species. Acid is added to such an extract to make it roughly decinormal, and it is then straightway heated as above without aeration. Success was obtained by the use respectively of twenty-four *Colias edusa*, twelve *Catopsilia argentea* and sixteen *Appias nero*, the amount of fluid being 70 c.c. in each case.

As a matter of fact a high dilution was used in all preparations of the crystalline product including those on a larger scale (4 ml. of acid for each mg. of pigment). This would doubtless be inconvenient for work on a really large scale, but it may be again remarked that it is exceptionally easy to separate small quantities of the product quantitatively from quite large amounts of mother liquor.

The nature of the dealings with the product as described in the last section was such that it was convenient to give the essential experimental details in course of the description. They do not call therefore for further reference here.

The sealed-tube products from uric acid

During the present research no serious attempt could be made to obtain these in a pure form. The experiments have been essentially qualitative in kind, chiefly intended to obtain information with regard to their yield of purple derivatives similar to that which is so characteristic of the natural coloured pterins. In all the preparations made the contents of the tubes to be heated have been kept uniform. Each has contained 2 g. of uric acid with 20 c.c. of distilled water, with the addition of 0·5 c.c. of sulphuric acid. This addition of acid has been empirical and perhaps unnecessary. The actual yield of yellow products appears just as great with water alone and their formation is certainly no acid effect, but by its combining with a portion of the ammonia produced hydrolytically the contents of the tubes after heating have a pH which does not rise above 7·5–7·6. An impression was early acquired that this was favourable in subsequent fractionations, and perhaps increased the yield of the purple product. The tubes were cooled in ice and salt before opening, as the pressure of CO_2 becomes high during the course of heating. This has always been maintained for 6 hr. at 190–195° C. Many preparations have been made, and steps in the fractionation of the products have been varied in detail. The yield of purple oxidation products by such fractions is described in § 3.

My grateful thanks are due to many who have generously contributed to my supply of coloured pierids. In particular I am indebted to Mr R. Winckworth, whose contribution was large and valuable, and to Mr N. D. Riley, Director of the Entomological Department of the British Museum, who assisted me not only directly, but also indirectly by making my wants known to others. I have received most welcome contributions and much kindness from the following: Professor G. Hale Carpenter of Oxford, Dr Karl Jordan of the Tring Zoological Museum, Major H. Blackiston, Chairman of the Museums Committee at Brighton, Mr René Oberthur of Rennes, Dr C. B. Williams, Head of the Entomological Department, Rothamsted, Mr H. M. Pendlebury of Kuala Lumpur, Mr W. Rait-Smith, Lt.-Col. F. C. Fraser and Mr G. M. Russell. The following dealers spent much trouble on my behalf: Messrs Newman of Bexley and A. Ford of Bournemouth. I have throughout received able technical assistance from E. J. Morgan.

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The photosensitivity of visual purple solutions and the scotopic sensitivity of the eye in the ultra-violet

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Previous measurements of the spectral variation of the photosensitivity of visual purple solutions have been extended into the far ultra-violet. The products have been found to be the same as in the visible.

Dark adaptation curves, produced by various observers, including one with an aphakic eye, were of the same type at 365 as at 546 mμ. The limiting scotopic sensitivity (the reciprocal of the potential retinal illumination in quanta/sec./sq. mm. which is just sufficient to excite vision) of normal eyes at 365 mμ is more than ten thousand times smaller than that to be expected from the photosensitivity at this wave-length, a fact which is attributed to the presence of light absorbing substances in front of the retina. The scotopic sensitivity of the aphakic eye was, however, as expected, indicating the absence of absorbing substances. Indeed the aphakic eye was practically as sensitive at 365 as it was at 546 mμ.

The limit of vision in the ultra-violet was accurately determined and, for normal eyes, found to correspond to the threshold of absorption of the lens, 309 mμ. The limit for the aphakic eye (no lens) corresponded to the threshold of absorption of the cornea, 298 mμ.

1. Introduction

The relation between the rate of bleaching of visual purple solutions by visible light and the sensitivity of the eye in the dark-adapted state (scotopic vision) has been described in a previous paper (Schneider, Goodeve & Lythgoe 1939). The rate of bleaching at any particular wave-length is governed by the photosensitivity, i.e. the product of the extinction coefficient α and the quantum efficiency γ.

The scotopic sensitivity of the eye (or the luminosity of the light†) is given by the reciprocal of the light intensity on the retina necessary to produce a standard response. The most accurate measurements of its relative values were made by Abney & Watson (1915). These were recalculated to a quantum basis by Dartnall & Goodeve (1937). It was found (Schneider *et al.* 1939) that the photosensitivity of visual purple corresponds exactly to the scotopic sensitivity of the eye on the red side of the spectrum, but on the blue side the scotopic sensitivity is less than the photosensitivity. This discrepancy was attributed to the presence of yellow substances in the eye which absorb light, and thus reduce the intensity of the light absorbed by the visual purple. Dartnall & Goodeve (1937) deduced from the relation between the curves that the optical density at 502 mμ of the visual purple in the retinae is not greater than 0·2.

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* Deceased.

† In previous papers the term 'scotopic luminosity' has been used in conformity with common practice. Its use, however, implies the existence of a 'standard eye'. While this is a useful concept for the visible part of the spectrum, the great variation in the sensitivity of different eyes for the ultra-violet makes it necessary to use a term to describe the eye rather than the light. A definition of the 'absolute scotopic sensitivity' is given later in this paper.

These methods have now been extended to the ultra-violet region.

2. THE PHOTOSENSITIVITY OF VISUAL PURPLE IN THE ULTRA-VIOLET

Hosoya (1937) found that visual purple was sensitive to ultra-violet light of wave-lengths 365, 313, 302 and 297 mμ. He measured the absorption as the visual purple was bleached, and with wave-lengths 365, 313 and 302 mμ the optical density increased with time of illumination. This is in agreement with the fact that indicator yellow, the product of bleaching (Lythgoe 1937, Goodeve, Lythgoe & Wood, unpublished) absorbs more strongly than visual purple at wave-lengths below about 420 mμ (depending on pH). Hosoya's results, however, do not give straight lines when analysed by the method of photometric curves. This means that the photoprocess in his case was not a simple one. Values of $\alpha\gamma$ could not be estimated as absolute intensities are not stated.

Method and observations for 405 *and* 365 mμ

The method of photometric curves has been fully developed in previous papers (Dartnall, Goodeve & Lythgoe 1936, 1938; Goodeve & Wood 1938) for cases of normal bleaching, i.e. where the optical density decreases with time of illumination. There it has been shown that if I_t is the light transmitted by a solution at time t, and I_f a constant final value of the transmitted light corresponding to the absorption of the bleached substance, then $\log_e (I_t/I_f - I_t)$ plotted against time gives a straight line whose slope m is a measure of the photosensitivity according to the equation $\alpha\gamma = \frac{m}{\phi}\frac{A}{I}$, where A is the area of the cell and I the intensity of the incident light. The correction factor ϕ is unity if no absorbing impurities are present in the solution and no absorbing products are formed in the photoreaction. It is found to vary very little over the range of I_t occurring in any one experiment, and thus may be considered as a constant.

The method can also be applied to cases in which the optical density *increases* with time of illumination, i.e. I_t is always greater than I_f. The integration of the original differential equation (equation (4), Dartnall *et al.* 1936) leads to the logarithm of the *modulus* of $(I_f - I_t)$, and one can therefore plot $\log_e (I_t/I_t - I_f)$ against time to obtain the slope m. This has been done for visual purple bleaching curves at 405 and 365 mμ, and straight lines have been obtained as shown, for example, in figure 1.

The apparatus and procedure for these wave-lengths were exactly as described in the previous paper (Schneider *et al.* 1939), except that a mercury-arc lamp was used and to the monochromator was added a violet filter for 405 mμ or a Wood's ultra-violet filter for 365 mμ. The results are shown in table 1.

The product of bleaching by 405 and 365 mμ was found to be 'indicator yellow', as is the case for longer wave-lengths (Lythgoe 1937). That there is no intermediate product of an appreciable life is shown by the fact that the photometric curves are straight lines (compare Dartnall *et al.* 1938). Many experiments made in these laboratories have shown that, when visual purple is bleached with visible light, there is a linear relation between the decrease in the visual purple band and the increase in that part of the indicator yellow band lying in the visible. That a linear relation also holds for bleaching at 365 mμ was shown by a separate experiment in which the bleaching was interrupted to allow measurements of the density at 502 mμ.

Method and observation for 254 mμ

The extinction coefficient of visual purple rises rapidly below 300 mμ as seen in figure 2. This absorption has been attributed to the presence of proteins (Krause & Sidwell 1938; Goodeve, Lythgoe & Wood, unpublished). It was shown by the following experiment that illumination with monochromatic light of wave-length 254 mμ causes a progressive destruction of the visual purple absorption band in the visible part of the spectrum and a growth of the indicator yellow band in the normal way.

Light from a mercury-arc lamp was rendered monochromatic at 254 mμ by a Hilger-Müller quartz double-monochromator and was focused through a hole in the wall of a light-proof hut on to the quartz cell containing the visual purple, the whole optical arrangement being essentially the same as before (1939). The absolute value of the incident light intensity, I, was measured with a thermopile. As the density at this wave-length is very high, i.e. the absorption is practically complete, and does not change appreciably on bleaching the visual purple (Goodeve, Lythgoe & Wood, unpublished), it was necessary to follow the change by interrupting the illumination from time to time and measuring the density at 502 mμ. These measurements were made on an apparatus described by Bayliss, Lythgoe & Tansley (1936). Comparison measurements were made with an unexposed solution in order to correct for the dark reaction which was appreciable in the 18 hr. required to bleach with 254 mμ to half density at 502 mμ. Density measurements on the illuminated solution at wave-lengths between 400 and 430 mμ taken along with those at 502 mμ, gave a cross-over point at 418 mμ (pH = 9·3) showing that indicator yellow is produced (Lythgoe 1937).

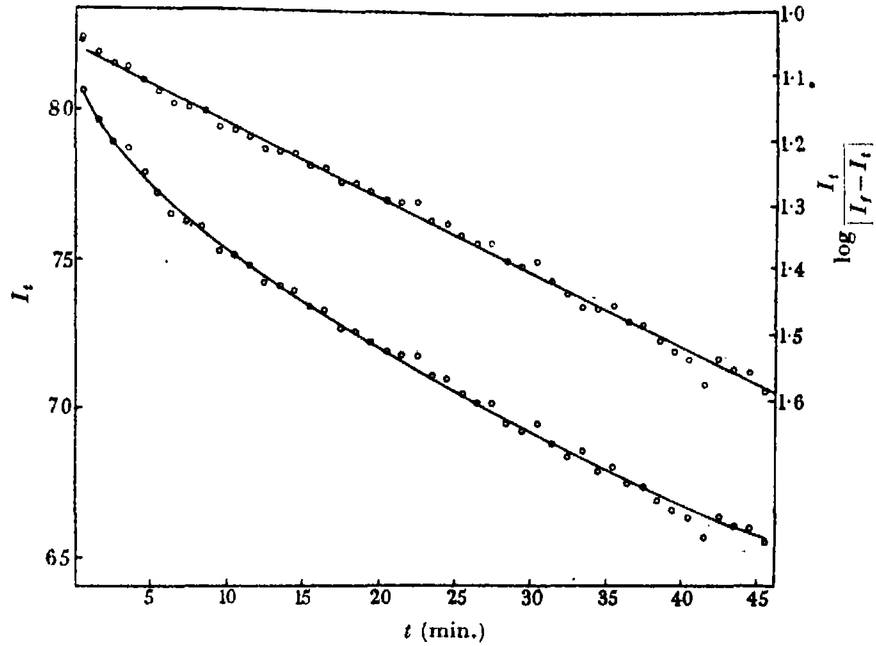

FIGURE 1. Showing the application of the method of photometric curves to cases where the optical density increases (transmitted light, I_t, decreases) with time of illumination. Example, visual purple solutions at 365 mμ. The scale on the right refers to the upper curve.

TABLE 1

pH 9·3, Temp. ~20°, area of cell 0·825 cm.^{2}

1 experiment number	2 initial density	3 final density	4 intensity $\times 10^{-13}$ quanta/sec.	5 slope $\times 10^3$ sec.$^{-1}$	6 ϕ mean	7 $\alpha\gamma \times 10^{17}$
			$\lambda = 405$ mμ			
12*a*	0·142	0·193	4·26	0·83	0·80	2·01
12*c*	0·328	0·377	1·41	0·23	0·70	1·92
15*c*	0·269	0·320	4·16	0·73	0·70	2·07
					Mean	2·0
			$\lambda = 365$ mμ			
11	0·353	0·478	1·16	0·184	0·60	2·2*
12*b*	0·266	0·458	1·07	0·194	0·60	2·5
15*a*	0·338	0·515	2·34	0·48	0·57	2·97
					Mean	2·6

* pH = 7.

Although the method of photometric curves can be extended to cover the above type of case, particular care is necessary in interpreting the value of the photosensitivity, $\alpha\gamma$, so obtained. Visual purple is normally defined as a light-sensitive chromophore with a maximum extinction at 502 mμ. The absorption at 365 and again at 254 mμ may be due to the same chromophore as that at 502. On the other hand, a light quantum responsible for bleaching by the latter wave-length may be absorbed by a neighbouring or even an entirely independent chromophore, which then acts as a photosensitizer, destroying the visual purple chromophore.

Both these mechanisms can be expressed in mathematical terms, but the accuracy of the measurements made here would not justify their reproduction. However, for the first case where the absorption at 254 mμ belongs to the visual purple chromophore the value of $\alpha_{254}\gamma$ is about $0{\cdot}5 \times 10^{-17}$ cm.^{2}.

Discussion

The mean values of $\alpha\gamma$ from table 1 and the values for 254 mμ are shown in figure 2 together with the curve of α for visual purple determined by Goodeve, Lythgoe & Wood (unpublished). The values of $\alpha\gamma$ for the visible region of the spectrum are included from the previous paper (Schneider *et al.* 1939). It will be seen from the figure that the accurate coincidence of the curves in the visible, indicating a constancy in γ, does not hold below 430 mμ. A maximum at about 360 mμ and a minimum at about 400 mμ is,

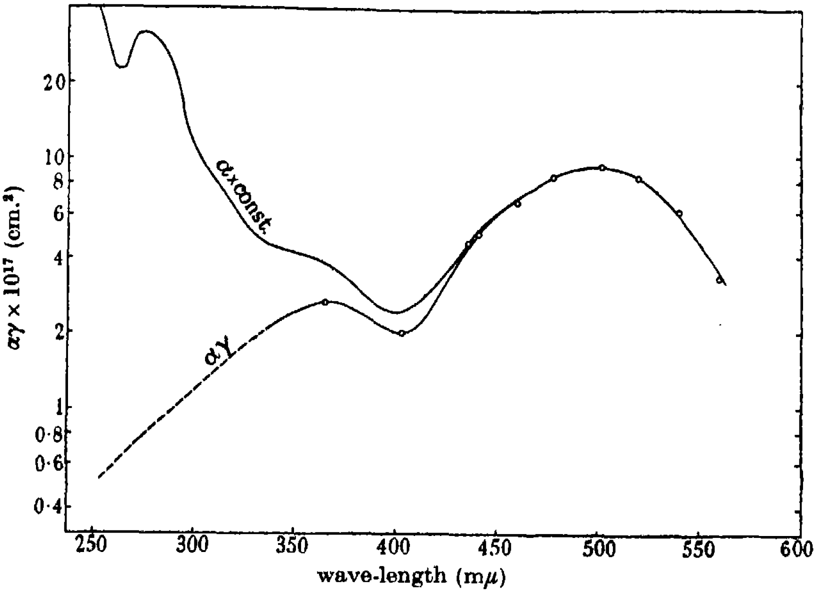

FIGURE 2. The photosensitivity, $\alpha\gamma$, of visual purple for visible and ultra-violet light (—o—o—) compared with the absorption curve of visual purple (upper curve).

however, common to both curves. This means that the absorption band at 360 mμ is closely connected with the visual purple chromophore and corresponds to a transition to a higher electronic level than that for the band in the visible.

There appear to be two possible explanations of the difference between the curves in the near ultra-violet. If we assume that the observed value of α is solely due to visual purple, the value of γ must be about one-half what it is in the visible. This would mean that the process of transformation of the absorbed light quantum into chemical energy necessary for the disruption of the visual purple chromophore is different, depending on whether absorption takes place in the visible or near ultra-violet band.

The other explanation is that the values of α for the absorption band of the visual purple chromophore at 360 mμ lie below the absorption curve shown. This would mean that visual purple preparations contain a photostable component absorbing in the ultra-violet. There are many possibilities for such substances. Broda recently (1941) has found a yellow phospholipin in solutions of visual purple, the light absorption of which increases with decreasing wave-length throughout the range concerned.

It is not useful to discuss the results in the far ultra-violet, except to say that here in addition strong protein absorption sets in.

3. SCOTOPIC SENSITIVITY IN THE ULTRA-VIOLET

From the experiments described in the previous section it appears that light of any wave-length down to 254 mμ causes the same photochemical change in visual purple, and therefore one would expect the retina to be sensitive to this range of ultra-violet light. It is known, however, that there is a sharp limit of vision between 302 and 313 mμ (Goodeve 1934) which has been attributed to the absorption of the crystalline lens. Preliminary experiments showed that the relative sensitivity of the eye to 365 mμ was very much less than would be expected from the photosensitivity of visual purple at this wave-length. Experiments were therefore undertaken to obtain quantitative information. The interest of these measurements was greatly extended by the observation of Gaydon (1938) that aphakic eyes are much more sensitive to ultra-violet than ordinary eyes.

Both chromatic and scotopic vision apparently occur throughout the range of 'visible' ultra-violet light, and the mechanism of vision appears to be the same as for ordinary visible light. At high intensities the ultra-violet light has colour, which is described by various observers as violet, blue, lavender grey, etc. As the intensity is reduced the colour disappears, leaving a grey image, such as is characteristic of scotopic vision at longer wave-lengths. A further characteristic of scotopic vision, the increase in sensitivity with time in the dark, is also found in the ultra-violet. In the experiments described below the rate of increase of sensitivity with dark adaptation was found to be the same for 546 mμ as for 365 mμ, and the curves are in all respects similar (see figure 4).

Apparatus

The apparatus was arranged as shown in figure 3 in a hut capable of being darkened. A quartz-mercury lamp *H* was focused on the slit S_1 of the glass monochromator (see Schneider *et al.* 1939). The slowly rotating shutter *C* gave flashes of 1 sec. duration every 5 sec. The emergent light from the slit S_2 having passed through the Wood's ultra-violet filter F_3, was focused by lens *L* on the eye of the observer. A circular aperture, 15 mm. diameter, in the screen *P* was thus uniformly illuminated, and formed the object for observation. The position of the observer's head was fixed by a biting device and observations were made with the field of vision centred on the red light spot, *R*, lying to the left of *P* and produced by the source *N* ($\lambda > 800$ mμ). The distance between *R* and *P* was such that the angle subtended at the eye was 10°. Observations were thus made using the parafoveal region of the retina 10° from the centre along the 180° meridian, in the temporal or nasal side of the retina according as the observer used his left or right eye. According to Wentworth (1930) the sensitivity here is not far below the maximum for the eye.

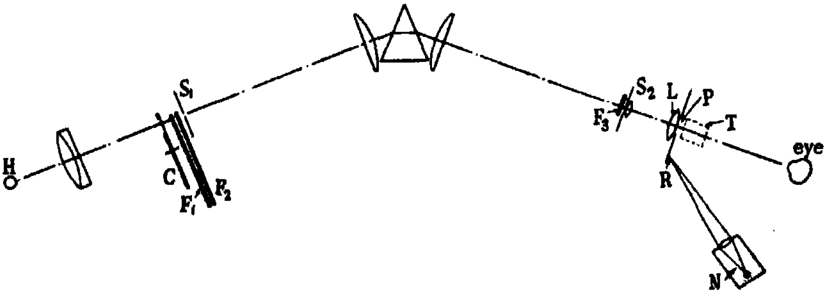

FIGURE 3. Arrangement of the apparatus for measurement of the scotopic sensitivity of the eye.

The intensity of the light was altered by means of two Ilford neutral wedges, F_1 and F_2, which had been carefully calibrated for both wave-lengths by a photocell and by a Hilger Spekker photometer. Their range of optical density, $D_1 + D_2$, was from 0 to about 4. The Wood's ultra-violet filter was in the first place used to absorb visible light scattered in the monochromator when using 365 mμ, but was kept in position for both wave-lengths because its low transmission (10^{-5}) at 546 mμ conveniently compensated for the high sensitivity of the eye to this wave-length compared with that at 365 mμ.

The total intensity of the light, N_0 quanta/sec., for the zero positions of the wedge filters was determined by placing a thermopile at T. With the ultra-violet filter in position it was not possible to measure the small intensity at 546 mμ with the thermopile, and it was therefore necessary to remove it for this measurement. Its optical density had then to be determined accurately; this was done by comparison with the wedge filters, a value 5·0 ± 0·1 being obtained.

Dark adaptation curves

Dark adaptation curves were obtained by the following procedure. The observer's eyes were first light adapted by observing for about 3 min. a white screen illuminated to about 10 f.c. He then took up his position and darkened the hut, the operator starting the stop-watch. Before starting, wedge F_1 was in the zero position, i.e. no absorption, and wedge F_2 in a position corresponding to a rather high density. As soon as the observer was ready with his eye focused on the red spot, the operator moved wedge F_2 slowly towards *lower* densities until the observer first saw the light flash in the lens L, whereupon he signalled by ringing a bell. The time was recorded and the density of the wedge F_1 now *increased* by two divisions on the wedge scale corresponding to an increase of D_1 of about 0·12 at 546 mμ and 0·29 at 365 mμ, the exact value being obtained from the calibration curves for F_1. (Wedge F_2 was left unmoved throughout the remainder of the experiment.) As the sensitivity of the eye increased, the flash again became visible, the bell rung, and the time recorded. The density of wedge F_1 was again increased by two scale divisions. This procedure was carried on for about 10 min., the increment in D_1 being altered in the last few minutes to one scale division.

One series of observations usually consisted of

3 min. light adaptation,
10 min. observation on 365 mμ,
3 min. light adaptation,
10 min. observation on 546 mμ,
3 min. light adaptation,
10 min. observation on 365 mμ.

For each experiment the values of D_1 were plotted against time. One series of observations is shown in figure 4. The curves are of the usual form, which has been attributed to the superposition of rod and cone adaptation. Kohlrausch (1922) and Hecht (1929) found one break only in the curve, but in many of the curves determined here there appeared to be a number of breaks which were only partly reproducible.

Calculation of energy thresholds

The number of quanta/sec., N, entering the pupil of the eye, is related to the number, N_0, measured by the thermopile, by the equation

$$N = 10^{-K_1}\frac{B}{A}\frac{E}{C}(1-r)(1-r')N_0 \times 10^{-(D_1+D_2)}$$

$$= 0{\cdot}068 \times 10^{-K_1}N_0 \times 10^{-(D_1+D_2)}, \qquad (1)$$

where A is the area of the light patch falling on the screen P (= 420 mm.^{2}), B the area of the aperture in P (= 176 mm.^{2}), C the area of the light patch at the pupil (= 270 mm.^{2}), E the area of the pupil (assumed dark adapted and equal to 50 mm.^{2}), r and r' the reflexion losses at lens L and at the cornea of the eye (assumed to be 0·1 and 0·05 respectively), and K_1 the optical density of the cornea and anterior chamber of the eye. The values of N_0 for 365 and 546 mμ were always close to $1{\cdot}2 \times 10^{12}$ and $3{\cdot}1 \times 10^{8}$ quanta/sec. respectively.

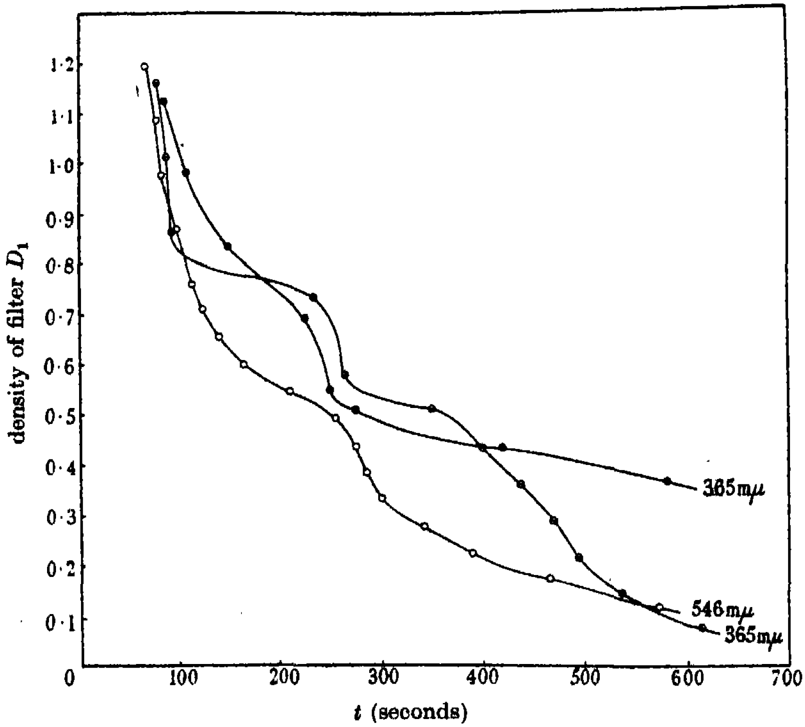

FIGURE 4. Typical curves showing the increase in sensitivity of the eye (decrease in threshold) during dark adaptation.

The intensity of illumination, J, of the retina, in terms of the number of quanta/sec./sq. mm. is related to N by the equation

$$J = N/a. \qquad \times 10^{-K_2}, \tag{2}$$

where K_2 is the optical density of the crystalline lens and posterior chamber and a, the area in mm.^{2} of the image on the retina, given by

$$a = B\left(\frac{f}{d}\right)^2. \tag{3}$$

Here f is the anterior focal length of the complete optical system of the eye and d the distance of the object from the first principal plane, this plane being about 1·5 mm. behind the cornea. In the apparatus described, $d = 328$ mm. whilst in the normal eye accommodated for this distance $f = 16{\cdot}1$ mm. (Gullstrand's value, see Southall 1937). It follows that for normal eyes a was 0·42 mm.^{2}, i.e. a circular patch subtending 2·6°.

The total optical density, K, of the eye, which includes the absorption of all of the different media of the eye through which the incident light passes before it strikes the light-sensitive part of the retina, is given by $K_1 + K_2$.

Let us define as potential retinal illumination J_p the intensity of illumination of the retina (measured in quanta/sec./mm.^{2}) in the absence of any absorption of light in the eye

$$J_p = J \times 10^{+K}. \tag{4}$$

J_p is the only quantity which can in practice be measured. As will be seen below (p. 393) however, J, and thus K, can be deduced from scotopic sensitivity values. It has been found that for wave-lengths above about 450 mμ the media of the eye are practically transparent, i.e. K is negligibly small. At 546 mμ the potential retinal illumination is therefore practically equal to the actual illumination.

The sensitivity of the eye to light of any wave-length can only be determined relative to some standard wave-length, but is proportional to the reciprocal of the light intensity on the retina which produces a given intensity of response. It is therefore convenient to define S_λ, the absolute scotopic sensitivity of the eye (see footnote p. 380) as the reciprocal of the potential retinal illumination which is just sufficient to excite vision. Therefore

$$S_\lambda = 1/J_p \quad \text{or} \quad \log_{10} S_\lambda = -\log_{10} J_p. \tag{5}$$

The value of S_λ is a characteristic of the eye depending only on the degree of dark adaptation and on the size of the retinal image. The effect of size or area of image on luminous thresholds has been studied by a number of observers including Piper (1903), Henius (1909) and Löhle (1929). At least for areas less than about 10′ in diameter the ability of the eye to see a light source depends only on N, the total number of quanta/per second entering the eye, and is independent of how these are distributed. For areas greater than a certain size, it seems to be independent of the area and to depend only on the intensity of retinal illumination (Löhle 1929). The intermediate region of area is the most convenient one for experiment. From the results of Piper (1903) and of Henius (1909) it appears that scotopic sensitivities measured for 2·6° should be multiplied by a factor of about 1·5 if they are to be compared with results obtained with areas of 1 sq. mm. (4°). It is considered at this stage better not to correct the results in this way, and it should be borne in mind that they are therefore to be associated with the retinal area of 0·42 mm.^{2} or 2·6°.

Observer with aphakic eye

The left eye of one observer was aphakic (i.e. without a lens) as a result of a traumatic cataract. For the observations he used a spectacle lens of +11·5 dioptrics (87 mm. focal length) placed 12 mm. from the cornea. The pupil was very constricted, but treatment every other day with atropine produced an aperture, equal to 15 ± 5 mm.^{2}. This was increased by the spectacle lens so that E (in equation (1)) for this observer was 20 mm.^{2}. The value of r' in this case was increased owing to the extra reflecting surfaces and slight absorption by the glass at 365 mμ. Tests showed that these effects could be accounted for by making r' equal to 0·15 at 546 mμ and 0·35 at 365 mμ.

In an aphakic eye the anterior focal length of the optical system is 23·2 mm. and the first principal lies 0·05 mm. in front of the cornea (Southall 1937). The composite optical system consisting of the aphakic eye and the spectacle lens placed 12 mm. in front of the cornea has an anterior focal length of 20·6 mm., and its first principal plane lies 1·1 mm. in front of the cornea. The size of the image a was therefore in this case 0·71 mm.^{2}.

Results and discussion

The scotopic sensitivity for six observers is given in table 2. The two values for each wave-length were from curves taken before and after that for the other wave-length, and in the cases where two sets for one observer's eye appear, the second was taken some weeks after the first. It is estimated that the limits of error of the absolute values of log S_λ are ± 0·3 for 546 mμ and ± 0·2 for 365 mμ arising out of accumulative errors in calibrating the filters. These, of course, are fixed errors and do not account for the differences in the results obtained by one observer. On the whole, however, the reproducibility of the results is good considering the difficulties in making observations using the parafoveal part of the retina. The average of each pair is used to determine S_{546}/S_{365}.

It is seen from table 2 that for most of the observers the values of log S_{546} after 600 sec. lie between −4·1 and −4·4, indicating that about 20,000

TABLE 2

			logarithm of scotopic sensitivity after dark adaptation of								
			150 sec.			400 sec.			(final reading after) about 600 sec.		
	observer	eye and age	$\log_{10} S_{546}$	$\log_{10} S_{365}$	$\log_{10} S_{546}/S_{365}$	$\log_{10} S_{546}$	$\log_{10} S_{365}$	$\log_{10} S_{546}/S_{365}$	$\log_{10} S_{546}$	$\log_{10} S_{365}$	$\log_{10} S_{546}/S_{365}$
A	(W. D. P.)	left	−4·7	− 8·6	+3·6	−4·4	− 8·1	+3·4	−4·1	−7·7	+3·4
		20		− 8·0			− 7·6			−7·3	
B	(E. E. S.)	right	−4·6	− 9·1	+4·9	—	− 8·7	—	−4·2	−8·4	
		27		− 9·9			− 9·2			−8·8	+4·4
B	(E. E. S.)	left	−5·1	− 9·2	+4·1	−4·5	− 8·8	+4·6	−4·4	−8·8	
		27		—			− 9·5			−8·9	+4·4
C	(G. C.)	right	−4·5	− 8·2	+3·6	−4·2	− 8·0	+3·6	−4·2	−7·7	+3·4
		27		− 8·1			− 7·7			−7·6	
D	(C. F. G.)	right	−4·6	− 8·2	+3·5	−4·2	− 7·6	+3·5	—	—	—
		34		− 8·1			− 7·9			−7·7	
D	(C. F. G.)	right	−4·9	− 8·8	+4·1	−4·6	− 8·3	+3·9	−4·4	−8·1	+3·9*
		34		− 9·2			− 8·8			−8·6	
E	(R. J. L.)	left	−5·5	−10·4	+4·9	−5·1	−10·0	+4·9	−5·0	−9·9	+4·8
		42		−10·4			−10·0			−9·7	
F	(A. G. G.)	left	−5·2	− 5·0	+0·1	−4·7	− 4·2	−0·3	−4·3	−4·0	−0·1
	(aphakic)	26	−4·7			−4·3			−4·0		
F	(A. G. G.)	left	−4·8	− 5·0	+0·3	−4·4	− 4·5	+0·1	−4·1	−4·0	+0·1
	(aphakic)	26		− 5·2			− 4·5			−4·4	

* Close to blind spot.

quanta/sec./mm.^{2} are necessary to stimulate vision at 10° from the fovea. Dark adaptation is generally not complete until much later than this, but the rate of increase becomes rapidly smaller. If the experiments had been prolonged it is probable that values of $\log S_{546}$ lying between $-3{\cdot}9$ and $-4{\cdot}2$ would have been obtained which would be in good agreement with a value of $-4{\cdot}0$ which has been calculated from Wentworth's measurements (1930). One of the normal eyes gave a low sensitivity ($\log S_{546} = -5{\cdot}0$), while the aphakic eye had the same value as the average.

A greater variation was found for the ultra-violet sensitivity. Three of the observers gave values of $\log S_{365} = -7{\cdot}6$, while two others were less sensitive at $-8{\cdot}7$ and $-9{\cdot}8$ respectively. (De Groot (1935) found differences up to about 100 : 1 between observers for this wave-length.) The anticipated high sensitivity for the aphakic eye has been confirmed and indeed it is seen that this eye was just as sensitive to 365 mμ as it was to 546 mμ. The constancy for all observers of the ratio S_{546}/S_{365} as adaptation proceeds indicates that the mechanism responsible for this type of vision is the same for both wave-lengths.

A comparison between these measurements of the scotopic sensitivity (after 400 sec. dark adaptation) and the photosensitivity of visual purple is made in figure 5, the former being 'linked' to the results of Abney and Watson at 546 mμ. The shape of the curve in the ultra-violet is only known very approximately, but the fact that there is a point of inflexion with normal eyes follows from observations made on numerous occasions in these laboratories. When a hydrogen discharge tube, which has an almost flat energy distribution curve, is used with the Hilger-Müller monochromator and the wave-length drum is turned towards the ultra-violet, one readily observes a rapid decrease in intensity near 400 mμ followed by a very much slower decrease until one approaches the limit (see below). Measurements of the *photopic* luminosity curve in the ultra-violet were made some years ago by one of the authors (C. F. G. unpublished) and clearly showed a marked flattening of this curve and a point of inflexion at about 350 mμ.

It is seen from the figure that *the relative scotopic sensitivity of the aphakic eye at* 365 mμ *is, within the limits of error, that to be expected from the photosensitivity of visual purple.* This indicates that in vivo as in vitro the photochemical process is the same in the ultra-violet as in the visible part of the spectrum, and emphasizes the fact that a photochemical change is the initiating process in vision.

The question now arises, what causes the greatly reduced sensitivity of normal eyes to the ultra-violet? The simplest explanation is that there is present in normal eyes a substance which strongly absorbs the ultra-violet but which is not present in the aphakic eye. In other words, in normal eyes the actual retinal illumination J by ultra-violet light is very small compared with the potential retinal illumination J_p. (The alternative, that γ_{365} is reduced without reducing γ_{546}, is difficult to imagine.) The lens and cornea of the eye are completely transparent to 365 mμ (Graham 1922; Abe 1927), and therefore the absence of the lens itself cannot account for the results with the aphakic eye. The presence of a yellow pigment in another part of the eye must therefore be assumed. For the eye with the lowest sensitivity shown in table 2 the density K (see equation (4)) would be 5·7 at 365 mμ, a value which is easily attained by a very thin layer of a normal absorbing chromophore. This yellow pigment may be a phospholipin (Krause 1934; Broda 1941), traces of it remaining in the extracted visual purple accounting for the difference between the α and $\alpha\gamma$ curves as mentioned above.

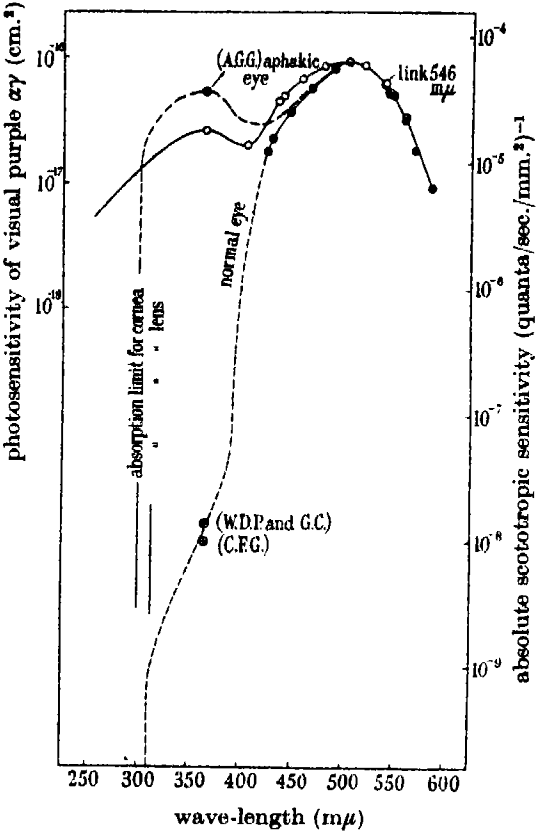

FIGURE 5. The absolute scotopic sensitivity (after 400 sec. dark adaptation) of visible and ultra-violet light for normal eyes and one aphakic eye, compared with the photosensitivity curve of visual purple (—o—o—). Abney and Watson's values for the scotopic sensitivity (—•—•—) are linked at ⊕ (546 mμ) to the present results.

The amount of yellow lipins in the various tissues of the eye is known to increase with the age of the observer (see Krause 1934, pp. 16, 29 and 203), a fact which fits in with the observations of Saidman (1933) and of de Groot (1935) that the sensitivity to the ultra-violet decreases with age.

The fundamental question why this yellow substance should disappear with cataract is an open one.

4. THE LIMIT OF VISION IN THE ULTRA-VIOLET

Previous determinations (Goodeve 1934) of the limit of vision in the ultra-violet were made with a mercury-arc lamp as light source, and it was found that the 313 mμ line was visible whereas the 302 mμ line was not. Vision at 313 mμ was apparently quite normal except that the object was in focus when only 4 in. from the eye (see also de Groot 1934).

More exact determinations of the limit have been made using a continuous light source (a hydrogen discharge tube) and a Hilger-Müller quartz monochromator. The slit widths were set to obtain a $\Delta\lambda$ of ± 1 mμ, and the size of the object, the exit slit, about 4 by 0·7 mm. The eye of the observer was dark-adapted for 5–10 min. (separate tests showed that the limit was practically constant after the first minute of dark adaptation). The exit slit was viewed foveally or slightly to one side from a distance of about 15 cm. When the monochromator drum was turned to shorter wave-lengths at an even rate by an operator, a progressive decrease of brightness was observed followed by a suddenly rapid decrease close to the limit. The limit itself was determined by turning at a slower rate and the readings were reproducible to ± 0·5 mμ. No measurement of the absolute light intensity was attempted and it is probable that, although the limit is sharp, it would move slightly to shorter wave-lengths if a higher intensity or a larger image were used.

The results are given in table 3.

TABLE 3

1935							
observer ...	C.F.G.		A.W.C.T.		R.J.McW.		J.W.G.
age ...	31		22		25		31
	R.E.	L.E.	R.E.	L.E.	R.E.	L.E.	R.E.
limit	310	310	314	311	309·5	309	311

1938–9				
observer ...	C.F.G.		W.D.P.	A.G.G. (aphakic)
age ...	35		20	26
	R.E.	L.E.	L.E.	L.E.
limit	311·5	311	308	300

Each result is the mean of from three to six measurements.

It is seen from the table that the aphakic eye is in a class by itself, its limit lying at 300 mμ. This presumably partly arises from a higher sensitivity of the retina, as found at 365 mμ. The more important factor, however, is probably the absence of the lens. Graham (1922) has examined a number of lenses both animal and human, and in all normal cases absorption was found to set in at 313·4 mμ. Less than about 5 % was transmitted at 309·5 mμ. The absorption of the cornea began at 298 mμ, while the vitreous and aqueous humours were transparent to much shorter wave-lengths. The agreement of these values with those of the limits of vision in table 3 is obvious, and it is therefore to be concluded that vision in the normal eye is limited by the sharp rise in the absorption of the lens, whereas vision in this example of an aphakic eye is limited by that of the cornea. The absorptions of the lens and of the cornea are due to those of the proteins which make up their structure. Therefore, although visual purple is sensitive to 254 mμ, vision at that wave-length is not possible.

The authors are indebted to the various observers, particularly Dr A. G. Gaydon, for their help, Dr Broda for his assistance in drawing up the paper and the Rockefeller Fund for a grant in aid of this research.

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On the incidence of the breeding season in mammals after transference to a new latitude

By the Duke of Bedford and F. H. A. Marshall, F.R.S.

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Ferrets, like other mammals with fixed breeding seasons in temperate latitudes, when transported to the southern hemisphere reverse their breeding seasons so as to conform to the new conditions. The change-over is very rapid, and consequently individual animals may experience two breeding seasons in one year. Ferrets taken to tropical latitudes where the seasonal conditions, at least as regards daylight duration, are comparatively uniform, experience sexual activity either at their former seasons or else come on heat irregularly.

Ruminants and some other mammals inhabiting tropical areas, when taken to temperate areas, do not react to the seasonal influence of daylight duration (in the manner of species inhabiting these countries), probably because they do not possess a developed mechanism of response to seasonal influences in their own countries where conditions in regard to light duration are comparatively uniform. This is shown more particularly for various species of deer.

It has been shown (Marshall 1936, 1937) that mammals when taken to a new latitude, as seen more particularly after transference to the southern hemisphere, are governed in regard to breeding periodicity by two main groups of factors: (1) internal ones depending upon the gonadal rhythm characteristic of the species (periods of reproductive rest and activity), and (2) external factors or responses to environment, the latter, as in the case of light, often acting exteroceptively through the nervous system. Under new conditions the two tendencies may for a time be antagonistic, but the animals usually become adjusted after a year or two or less. A similar general statement may be made about birds, and in the case of species transferred from the southern to the northern hemisphere the known facts have been set out and summarized by Baker & Ranson (1938). In the present paper certain further data are presented concerning the sexual periodicity of mammals removed from one latitude to another.

Carnivores. The researches of Bissonette (1932) and others show that ferrets, if acted upon by electric light or ultra-violet irradiation in the winter during what is normally their anoestrous or non-breeding season, may be made to come on heat and remain on heat for a prolonged time. It was to be expected therefore that such animals would similarly respond if transferred to the southern hemisphere. That this actually happens has been shown by Mr G. N. Murray of Onderstepoort, Pretoria, to whom we are indebted for the records. Ferrets which arrived at the Zoological Gardens at Pretoria in October 1937 (in the South African spring) bred immediately after their arrival. An adult female which was then taken to Onderstepoort had a litter of three on 9 February 1938, after having been on heat in the later part of 1937. In December 1937 another batch arrived from Europe at Onderstepoort and immediately came on heat and were shortly afterwards served. Two had litters of seven each on 13 March 1938, and one had a litter of five on 15 March. When the ferrets arrived they all had their long winter coats, but by the end of March they had lost their long hair and had short smooth coats. The last available record stated that two ferrets were obviously pregnant at nearly full term on 24 May 1938.

Mr S. Anderson, to whom we are likewise indebted, has reported on the breeding periodicity of ferrets imported into Kenya where the conditions are tropical and there is not the daylight variation of Britain or South Africa. He states that during the first year after importation the ferrets commonly experience oestrus at the normal British time. In addition, certain individuals may come on heat towards the end of the year (the anoestrous season of Britain) so that two breeding seasons may occur in one year, a condition which never occurs with ferrets kept at the same latitude nor with any wild member of the family of Mustelidae. On the other hand, certain individuals may breed at any times, possibly in response to local conditions other than light, some of these times being synchronous with the British times and some with months when ferrets do not breed in Britain.

With other carnivores there are few records of sexual periodicity in transported animals. It is known that lions and leopards inhabiting tropical areas have no regular breeding seasons (Shortridge 1934) so it was to be expected that when they bred in Europe they would do so irregularly. That this is so has been shown by the animals at the gardens of the Royal Zoological Society at Dublin, where the larger carnivores have been bred very successfully but not in response to seasonal change.

On the other hand, in the hunting dog of the Cape (*Lycaon pictus*) the records of the Dublin gardens (Cunningham 1905) show that there was a tendency at any rate to reverse their South African time for sexual activity. Thus Cunningham states that a female at Dublin had pups five times in January, but once in November and once in May. The period of gestation is about 80 days. The oestrous periods were usually in the autumn. In Cape Colony *Lycaon* is said to breed in the South African autumn, April being a usual month for the young to be born, but they might be born later (Lydekker 1926; Shortridge 1934).

Ruminants. The tendency on the part of the breeding organs to respond to increases of light, although very general among animals of all kinds, is not firmly established and is far from universal. Thus, as is shown in the previous communications, the response in ruminants where it exists must be to a decrease rather than an increase of light unless indeed it is the cumulative effect of an increase over a previous period. It has been shown, however, that with deer belonging to temperate countries and with regular breeding seasons there is a complete change-over in periodicity when the animals are taken from the northern to the southern hemisphere. This reversal is fairly rapid, taking about two years, and the onset of the breeding season is accelerated during the period when the change-over is taking place.

With deer and other ruminants inhabiting tropical countries the effects of removal to England are different. These appear to possess no adaptability, that is to say, they have no capacity to breed at appropriate seasons in England or at times when the native deer are sexually active. Moreover, tropical deer with breeding seasons in their own countries are as unadaptable as those which breed at all times of the year.

The sambur (*Rusa unicolor*), which inhabits the plains and forests of Ceylon, ruts in October and November in the plains and probably at any time of the year in other places according to Lydekker (1924). In the park at Woburn Abbey, where a herd has been kept, sambur could rut at any time of the year.

The axis or spotted deer (*Axis axis*) of India and Ceylon ruts at any time of the year in India (Heape 1900). They do the same at Woburn, where they have been kept in a herd of a hundred to two hundred and fifty for forty years in the park.

Reeve's muntjac (*Muntiacus reevesi*) of southern China and the common Indian muntjac (*Muntiacus muntjac*) are similar. The former lives in considerable numbers in the woods at Woburn, some being at complete liberty outside the Park. The Indian muntjac was kept at Woburn formerly for a good many years and the breeding habits of these deer were like those of the smaller species, that is to say, they bred at any season.

The hog deer (*Hyelaphus porcinus*) of India, Burma and Siam is recorded as rutting in September and October (Lydekker 1898, 1924), but it is unlikely that this occurs in the latitude it normally inhabits owing to any change in the duration of light. At Woburn the deer, which form a herd of fifty to sixty breed at all seasons.

The Javan rusa deer (*Rusa hippelaphus*), which form a herd of twenty at Woburn, have consistently produced their young in late autumn at the same time as they are believed to do in Java. This of course is a quite abnormal time for any deer which inhabit temperate countries.

The thamin (*Rucervus thamin*) in Upper Burma ruts in March to May, the young being born in October or November (Lydekker 1924). At Woburn, where there was formerly a small herd of about twenty, the rut was at the same season as in the natural state.

The nilgai (*Boselaphus tragocamelus*), a large antelope in the Indian peninsula, ruts from March to May (Heape 1900). In the park at Woburn, where a herd of thirty or forty has been kept, approximately the same breeding season is maintained, and the young are produced in winter.

It would thus appear that animals imported from tropical countries tend to maintain their internal sexual rhythm, since they do not respond to those differences in daylight to which the species inhabiting Europe or other temperate zones respond. Furthermore, it would seem probable that the explanation as to why tropical species do not so react lies in the fact that owing to comparative uniformity of conditions in their own countries they have never, so to speak, acquired a capacity to respond to variations in light duration or intensity in the manner characteristic of so many animals living under seasonally changing conditions.

According to Bodenheimer (1938) gazelles of various species kept in the Zoological Gardens at Cairo retain their normal breeding seasons although there is a general uniformity in external conditions.

As possibly relevant to this subject it is to be noted that guinea-pigs do not react to changes in light under experimental conditions in the same way as ferrets and so many other animals (Dempsey, Myers, Young & Dennison 1934), their sexual rhythms being but little affected. This may be connected with the fact that the ancestral guinea-pigs or cavies, like the ruminants referred to above, are tropical animals living under comparatively uniform equatorial conditions, especially as regards light, and so do not possess the capacity to respond to those seasonal changes which are the external factors fixing the periods of breeding in animals living away from the equator.

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The influence of selection on the preferendum of a Chalcid (*Microplectron fuscipennis* Zett.) and its significance in the biological control of an insect pest*

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The preferred temperature of *Microplectron fuscipennis* Zett., a Chalcid parasite of the European spruce sawfly, has been investigated with special reference to (1) changes produced by selective breeding, and (2) the significance of such changes in the biological control of insect pests.

The average preferendum of both sexes is between 21·2 and 22·8° C, with two minor peaks, one at 15° C and another at 9° C. In midsummer three groups preferring temperatures at 25, 15 and 9° C are nearly equal. Such changes were due to the selection of breeding stock and not related to seasonal or previous temperature experiences.

Within four inbred generations a line was established in which most of the individuals preferred the low temperature selected by the parents. So far, two groups have been isolated; one preferring a temperature of 25° C and another of 9° C.

1. Introduction

Most poikilothermal animals, when offered a wide range of temperatures, show a definite preference for certain temperatures. If insects are placed in an apparatus designed to provide a choice of numerous temperature conditions, they will remain active for some time but finally come to rest within a certain temperature zone. The preferred temperature thus apparently chosen by the insect is referred to as the *preferendum*.

The fact that the preferendum of terrestrial insects is profoundly variable is a characteristic of most reports on their behaviour in a gradient of temperature. Marked variations have been found not only between different groups and the various stages of development, but to some extent between the different areas within the geographic range of one species. It was, however, the detailed studies of Herter (1924) working with the ant, *Formica rufa* L., and of Bodenheimer and Schenkin (1928) with the beetles, *Tribolium confusum* Duv. and *Rhizopertha dominica* F., that first showed the differences in preferred temperature associated with (although not definitely dependent on) previous temperature experiences and general external temperatures. Their work has been extended to other species by many authors, of whom the most outstanding are Henschel (1929) and Totze (1933). For a more complete review of the literature the reader is referred to Fraenkel and Gunn (1940).

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In some cases, especially among those in which the reported changes are more conspicuously evident, it is clear that the shifting nature of the preferendum is related to factors other than those of previous temperatures and general external conditions. Bodenheimer and Schenkin (loc. cit.) have shown differences seemingly dependent on the external temperature. They failed, however, to find any relation between the preferendum and the average monthly temperatures, and could not explain the phenomenon. No one seems to have carried this investigation further to determine if the variations may be due to acclimatization, as were the differences in the chill-coma temperatures of several insects investigated by Mellanby (1939), or to some form of adaptation related to inherent differences within the parental stock.

The latter seemed to be of particular significance in connexion with the laboratory propagation and field establishment of the Chalcid parasite, *Microplectron fuscipennis* Zett., on the European spruce sawfly (*Gilpinia polytoma* Htg.) in Canada. This cocoon parasite, originally introduced from Europe, has been one of the most outstanding agents utilized by the Dominion Parasite Laboratory in the biological control of the sawfly (vide Baird 1939). Following initial liberation of laboratory-reared parasites in a number of widely separated areas, recovery studies indicated much more successful establishment and increase in certain areas than in others. Analysis of temperature and general ecological field data, when considered in the light of preliminary laboratory tests, indicated that these differences might be associated with the preferred temperature behaviour of the parasite.

It therefore seemed desirable to study the changes in preferendum shown by *Microplectron fuscipennis* Zett. first, in order to determine the nature of the factors involved, and secondly, to obtain information from which it might be possible to establish in the laboratory strains more or less specially adapted to different regions in the sawfly-infested areas. Hence, the present work is an attempt to determine the possible existence and application in pest control of intraspecific groups in which, to some extent, individuals might be isolated by means of temperature preferences.

2. METHODS AND MATERIALS

The methods employed in the determination of the preferendum are very varied. Those of more frequent use in the study of insect behaviour have involved the establishment of a continuous temperature gradient in which the animals may group themselves at any chosen temperature. The apparatus usually employed has been modelled after Herter's (1924) 'temperaturorgel' and consists essentially of a glass-covered, rectangular, metal trough, heated at one end and cooled at the other. Numerous variations of this type of apparatus have been cited by Fraenkel and Gunn (1940).

The apparatus used in the present investigation was of the same general construction. It consisted of a well-insulated metal trough, one end of which projected into a cooling cylinder and the other into a warming chamber. The trough was made from bars of copper joined to form a rectangular groove 38 in. long with a double strip of glass forming the roof. One end of the trough projected into a heating box, the temperature being regulated by means of a Dekhotinsky thermoregulator. The opposite end was immersed in a cooling solution provided with a simple stirring device. Holes were bored at intervals of 1 in. along one side through which were inserted thermocouple points. Although 38 points were set up, during the experiments only five were used; the remainder served mainly in testing the apparatus. The temperature along the various sections of the gradient was obtained by interpolation from a graphical plot of the five readings. Figure 1 shows examples of the temperature gradient in the trough during three experiments.

The routine followed in the handling of the experimental Chalcid flies was similar throughout, and therefore needs no special consideration. The gradient was first allowed to reach a steady state. A small number of *M. fuscipennis* Zett. was then placed in the centre of the trough. For a period of 2–3 days records of their position in the trough were taken at approximately 3 hr. intervals. Before being placed in the trough all the flies were kept at 23° C for at least 24 hr. In all but the field-recovered stock the immature stages were reared at that temperature.

During the experiments, the flies would wander almost from end to end of the gradient in temperatures from 5 to 40° C. In only a few cases did any of them come to rest for more than a few minutes outside of the temperature range for the group. The temperature at which the flies react violently to escape is 35–38° C. At 45° C and 80 % relative humidity they die in from 9 to 10 min. when transferred from 23° C.

The region of temperature in which the individuals congregate is referred to in the present study as the *preferendum* following the translation of *Vorzugstemperatur* by C. B. Williams (vide Bodenheimer and Schenkin 1928). As pointed out by Bodenheimer and Schenkin (1928) and later by Fraenkel and Gunn (1940), this does not necessarily imply that the preferendum can be stated as fractions of a degree. It is considered here as a zone in which 50–80 % of the individuals occur.

The data obtained from the different classes of *M. fuscipennis* Zett. tested have not been treated statistically. The mean preferenda with their standard error, although of little comparative value, have been included in a few cases, since they provide some measure of the error and relative variability of the groups. In order to show the general features of the distribution of each group, the results are presented in the form of frequency polygons, in which frequency in percentage is plotted against temperature.

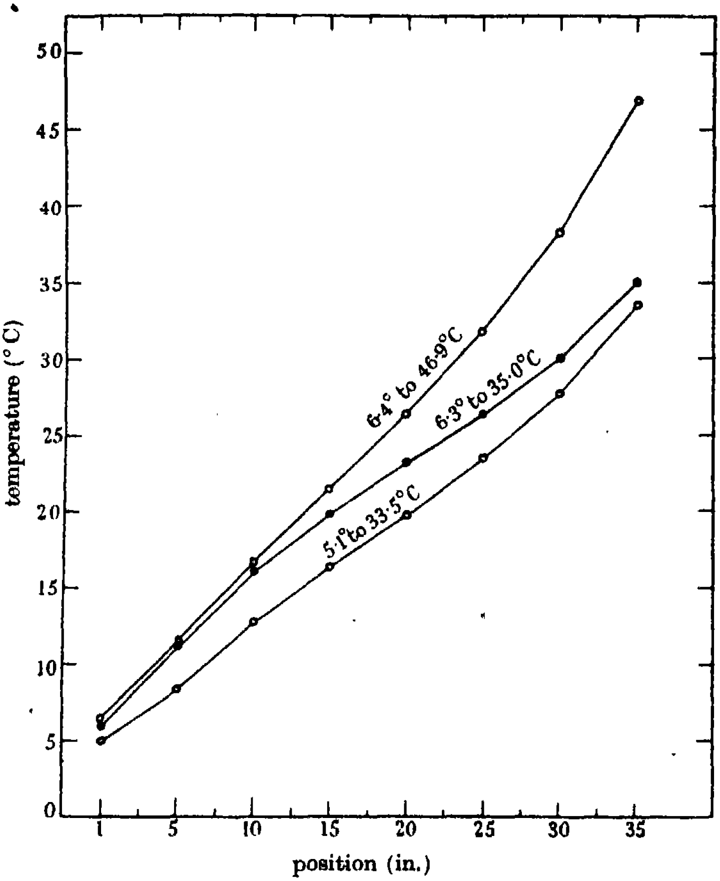

FIGURE 1. Examples of the temperature gradient in the trough. The maximum and minimum temperature range is indicated above each line.

3. RESULTS OF OBSERVATIONS AND EXPERIMENTS ON THE PREFERENDUM OF *MICROPLECTRON FUSCIPENNIS* Zett.

(*a*) *The* normal *preferendum of laboratory reared adults*

Although, as will be shown later, the preferendum of *M. fuscipennis* Zett. is subject to considerable variation, it would seem advisable, before dealing with such changes, to consider what might be regarded as an *ideal* or *normal* preferred temperature for this species. This has been done by testing in the thermal gradient random samples of laboratory-reared stock (reared at 23° C) during the mid-period of their breeding season. The data obtained from these individuals, virgin females, mated females and males, are tabulated in table 1. Their distribution in the gradient is shown graphically in figure 2.

TABLE 1. PREFERRED TEMPERATURE RECORDS OF NORMAL LABORATORY STOCK REARED AT 23° C

	virgin females	mated females	males
range of temp. gradient	6·1–47·1	6·4–46·9	5·7–34·8
number of observations	762	481	454
number of hours in gradient	45	45	47
percentage *observed in zones* between temp. given:			
5·1– 7·5	5·4	1·1	0·9
7·6–10·0	2·1	2·1	3·1
10·1–12·5	2·6	0·2	3·0
12·6–15·0	4·3	1·9	5·5
15·1–17·5	11·1	13·1	8·1
17·6–20·0	5·4	6·9	12·8
20·1–22·5	9·6	11·6	16·5
22·6–25·0	14·2	36·8	21·8
25·1–27·5	27·8	14·8	12·3
27·6–30·0	8·8	7·1	14·7
30·1–32·5	6·8	2·3	1·1
32·6–35·0	1·4	1·7	0·2
35·1–37·5	0·4	0	0
37·6–40·0	0	0·4	0
mean preferendum	$21{\cdot}70 \mp 0{\cdot}120$	$22{\cdot}84 \mp 0{\cdot}119$	$21{\cdot}25 \mp 0{\cdot}170$
percentage flies within 20–30° C range	60·5	70·3	65·3

The preferendum may be seen in figure 2 to be rather sharply defined and uniformly constant between 21 and 28° C for all three groups; 60–70 % of the flies congregated between 20–30° C. The average preferred temperature is 21·2–22·8° C. The differences between the means is not statistically significant. Although in all three there is a marked modal distribution at 25° C, the curves are not symmetrical. Each has a distinct negative skewness in which there appear to be two minor peaks in the vicinity of 15 and 8° C. The significance of the lower modes is referred to later.

(*b*) *Changes in the preferendum during the laboratory breeding season*

The first noticeable change from the *normal* preferendum of *M. fuscipennis* Zett. was observed when flies were tested at various periods during the active breeding period. In the rearing of this parasite for release, intensive laboratory propagation begins in May and continues until September. It is, therefore, possible to remove adults for preferred temperature tests at various periods during the summer. This was done in May, June and August, the complete data of which are shown in table 2. In figure 3 are shown the curves of percentage frequency plotted against temperature.

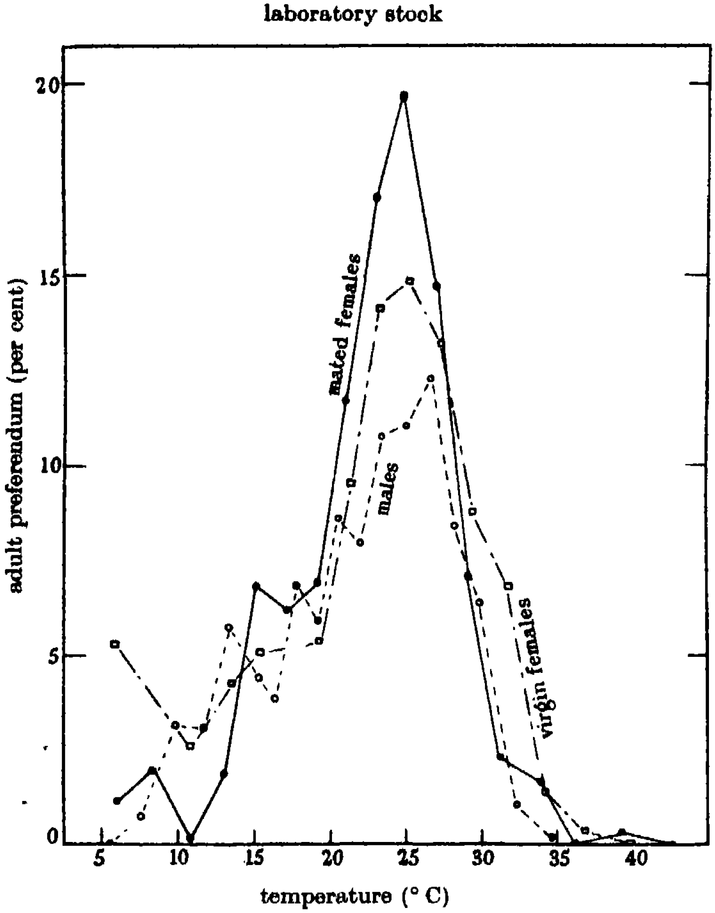

FIGURE 2. Preferred temperature of normal laboratory-reared *M. fuscipennis* Zett. Note the narrow preferred temperature range and the appearance of a somewhat trimodal distribution. Mated ♀♀ = 481; virgin ♀♀ = 762; males = 454.

It is clear from these results that there is a more or less general shift to the lower temperatures as the breeding season advances. The narrowest range of the preferendum is shown by the females reared in May, most of which preferred a temperature of between 17 and 23° C. Those reared less than 3 weeks later began to prefer a wider zone in the temperature gradient with a noticeable preference of some individuals for temperature near 15 and 8° C. Those reared in August show the greatest preferred temperature range. At that time, almost an equal number of females preferred the 25 and 8° C zones with only a slightly smaller number preferring 15° C.

Differences in the preferendum of this species as shown by the curves in figure 3 are of particular significance, in that although they are somewhat variable, they do not represent a direct shift of the *normal* curve toward the lower temperature of the gradient. Instead of a direct shifting of the almost unimodal May curve to the right, there is a marked reduction of the number preferring the higher temperatures and the establishment of a distinctly trimodal distribution in August.

TABLE 2. TEMPERATURES PREFERRED BY FEMALE FLIES REARED IN THE LABORATORY DURING AN AVERAGE SUMMER BREEDING SEASON

	laboratory-reared adults emerging on		
	9 May	1 June	3 Aug.
range of temp. gradient	6·0–34·5	6·4–38·8	5·9–33·6
number of observations	410	481	641
number of hours in gradient	63	45	56
percentage observed in zones between temp. given:			
5·1– 7·5	0	1·1	11·2
7·6–10·0	0·2	2·2	4·8
10·1–12·5	3·9	0·2	3·4
12·6–15·0	1·0	1·9	2·5
15·1–17·5	11·9	13·2	7·3
17·6–20·0	24·1	6·9	7·9
20·1–22·5	30·1	11·6	4·7
22·6–25·0	10·0	36·9	27·4
25·1–27·5	13·9	14·8	9·2
27·6–30·0	2·8	7·1	14·0
30·1–32·5	1·9	2·4	4·7
32·6–35·0	0·2	1·7	2·9
35·1–37·5	0	0	0

The most obvious explanation for such changes in the preferendum, since standard conditions were maintained throughout, was in the selection of adults for breeding. During the winter and until May the source of adults used for test purposes was a relatively small maintenance stock of closely inbred females. Thus the parents selected for breeding in each generation were those which emerged first from their host; the remainder being discarded. From May to the end of August large-scale propagation was conducted (approximately 60 million parasites per month). During this period the parents were outcrossed and all emergents were used in each succeeding generation. Thus, in May, the adults used for thermal gradient tests were from a limited group of a selectively inbred stock. Later in the season the flies were taken at random from a much larger group in which all possible segregants would be present. Thus it would seem that indirect selective breeding at 23° C over a period of successive generations reduces the number of individuals preferring certain low zones of temperature, but when random breeding is introduced there reappear all preferred temperature groups which are usually present in the normal population.

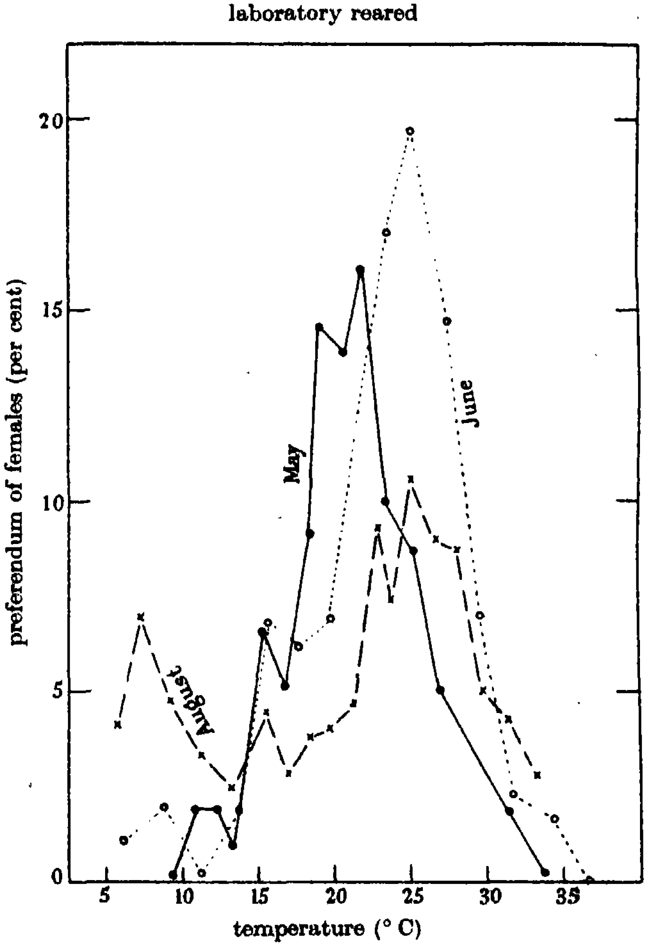

FIGURE 3. The preferenda of females bred continuously in the laboratory at 23° C. Tests were made in May, June and August. May = 410; June = 481; Aug. = 641.

(c) *Changes in the preferendum induced by selective breeding*

Before considering further the changes in preferred temperature illustrated in figure 3, it was regarded as possible that similar changes in the preferendum might be induced by the formal application of selective breeding methods. To do this females were selected from a group forming the lowest mode in figure 2; those congregating at 6–10° C. The progenies of this first selected generation were placed in the thermal gradient, and the parents of the second and subsequent generations selected and tested in the same manner. In 1939 time permitted of only two such selections.

During the 1940 season the selective breeding experiments were continued, beginning as before with selections from the normal laboratory stock. This line has now reached the 12th selectively inbred generation. Since, however, the data from each after the fourth generation are almost identical, only the first four and the last are shown in table 3. The changes in preferendum are illustrated graphically in figure 4.

TABLE 3. CHANGES IN PREFERRED TEMPERATURE DUE TO THE INFLUENCE OF SELECTION. THE RECORDS OF ORIGINAL PARENTAL STOCK ARE SHOWN IN TABLE 1

	1939 selected generations		1940 selected generations				
	1st	2nd	1st	2nd	3rd	4th	12th
range of gradient	5·7–35·8	6·0–35·0	6·3–35·8	6·0–32·0	6·0–33·2	6·0–34·0	4·7–32·9
number of observations	726	1050	637	820	945	878	431
hours in gradient	52	50	67	72	68	72	50
percentage observed in zones between temp. given:							
5·1– 7·5	0	9·4	0·3	5·4	4·2	9·5	9·7
7·6–10·0	0·8	17·4	6·6	8·0	26·3	30·7	28·4
10·1–12·5	9·3	8·6	1·9	7·8	7·9	10·6	13·1
12·6–15·0	7·7	17·3	9·7	9·1	6·9	7·9	9·5
15·1–17·5	8·4	5·0	9·1	10·7	9·6	5·0	16·3
17·6–20·0	9·2	6·4	7·8	12·2	8·0	7·9	4·9
20·1–22·5	8·3	7·4	21·0	19·3	9·9	6·1	10·5
22·6–25·0	9·2	11·2	23·4	9·3	11·8	7·2	4·8
25·1–27·5	23·6	10·7	9·9	12·4	5·6	7·4	1·4
27·6–30·0	18·9	3·0	9·7	4·1	6·5	1·6	0·9
30·1–32·5	3·2	3·1	0·5	1·7	2·5	4·4	0·5
32·6–35·0	1·4	0·5	0·1	0	0·8	1·7	0
35·1–37·5	0	0	0	0	0	0	0

In both series it is clearly noticeable that the flies even in the first selected generation contained fewer individuals which congregated at the higher temperatures. Although the large peak at 25° C shown by the original parental stock was still present, it was much reduced, representing not more than 50 % of the flies. In the subsequent selected generations a further and continuous preference for the low temperatures is observed, with a corresponding flattening of the curves to the right of the new modes.

It may be noted by a comparison of the curves given in figure 4 for the original parental stock in 1939 and the 4th selected generation in 1940 that there has been an almost complete reversal of the *normal* curve. In both there was a very pronounced modal point in the *normal* stock at 25° C, whereas in the selected stock it was between 8 and 9° C. The peak at 25° C, although becoming smaller in each selected generation, is persistently present in all the curves. The group preferring the 15° C zone, on the other hand, appears to be somewhat variable with respect to the number of individuals it contains and its position in the gradient.

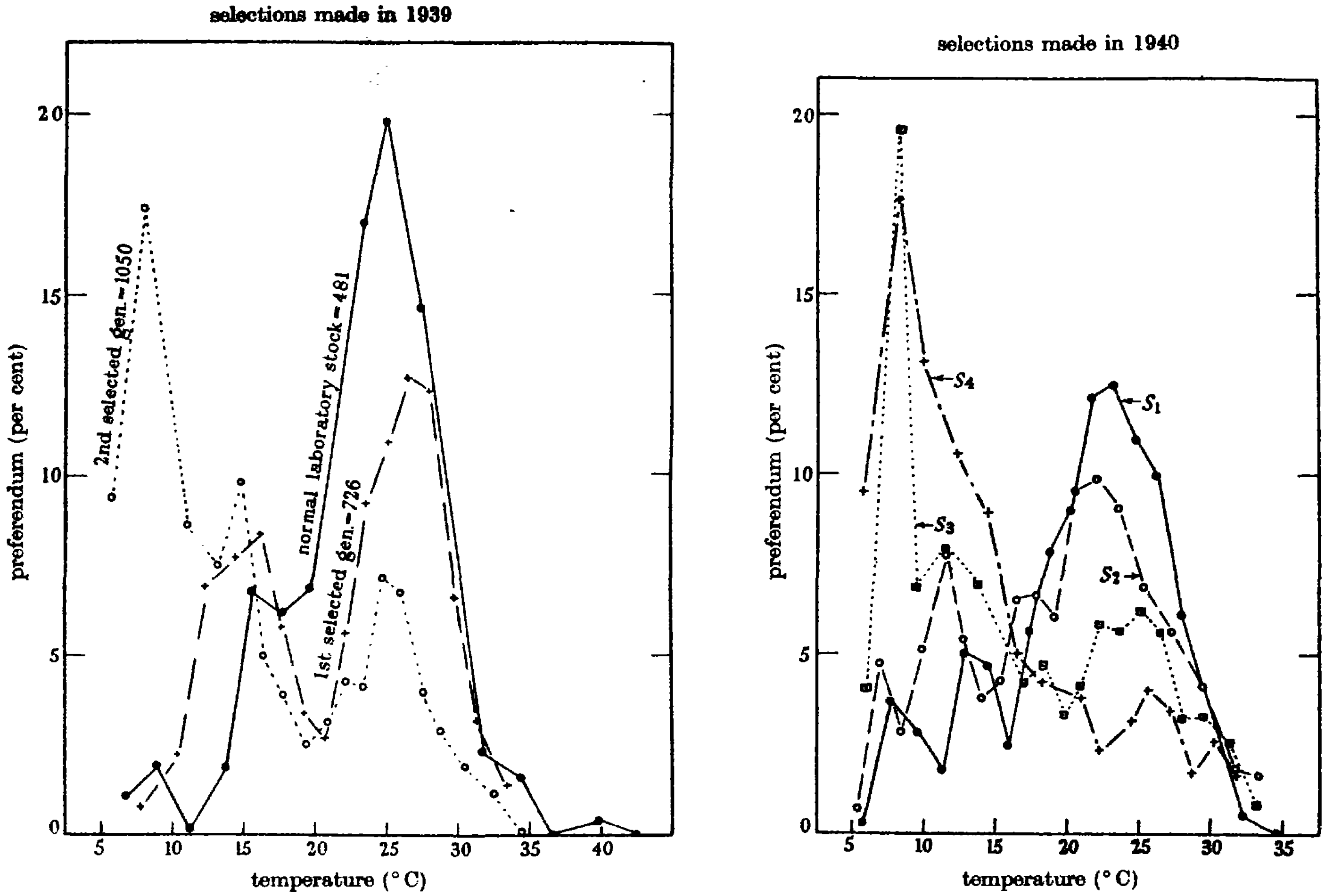

FIGURE 4. Changes in the preferendum produced by the selective breeding of 1939 and 1940 laboratory reared *M. fuscipennis* Zett. $S_1 = 637$; $S_2 = 820$; $S_3 = 945$; $S_4 = 878$.

Thus, in duplicate sets of experiments it has been possible by intentional selection to change the preferred temperature of this species. By the direct application of such practices as are commonly used in animal breeding a line has been established which has a distinctly lower preferendum than is shown by *normal* laboratory stock.

These observations provide further evidence to support the explanation suggested for the changes in preferendum observed during laboratory propagation; namely, that individuals of this species may be isolated into groups by differences in temperature preference. The number of groups present, however, is a matter requiring further investigation. Although it has been shown that there are two well-established preference groups at approximately 23–25° C and 8–10° C, it seems that others may be present. The necessity of maintaining standard conditions has limited the number of selections used in the thermal gradient tests. The significance of the group represented by the 15° C peaks, therefore, although noticeably present in almost all the curves shown, is not definitely known.

4. CONCLUSIONS

The position of the preferendum has been shown, both by evidence provided in the literature and from the results of the present experiments, not to be a fixed point, but to be variable. Many workers have found that the preferred temperature of different insects is not always the same, and they have usually considered the changes as seasonal or due to the environmental conditions of the insects just previous to the experiments. No one seems to have investigated the possibility of altering the preferendum by a method of selective breeding in the laboratory and thus relating the changes to inherent differences within the species.

The changes of preferendum shown by *M. fuscipennis* Zett. are not related to seasonal or previous temperature experiences, since in these studies the adults were treated in exactly the same manner, using one piece of apparatus under standard, uniform conditions. Although certain changes were observed during the summer season of laboratory propagation, these were due to differences in the method of selecting breeding stock. During the early part of the season the females used for breeding purposes were unintentionally selected for high-temperature preferences and thus showed an almost complete mean preference for temperatures between 21 and 23° C. Later in the season when breeding stock was taken more or less at random the change was not a direct shift of the *normal* preferendum to the lower temperatures but an establishment of three groups at 25, 15 and 9° C. Those present in each group were almost equal in numbers when parents were taken at random for propagation.

A direct attempt to change the preferendum by intentional selection gave similar results. The progeny of selected females preferring 6–10° C congregated in groups similar to those produced by the random use of breeding stock. By continuing to select those preferring the low temperatures, within four inbred generations a line was established in which over 40 % of the individuals preferred a temperature below 10° C as compared to 3 % in the parental, *normal* stock. The two groups at 25 and 15° C were still present, but they were represented by only a few individuals.

Thus in normal propagation, groups have been detected which, by selective breeding, have been more or less isolated into strains by differences in their temperature preferences. At least two groups have been isolated in this manner, one preferring a temperature of 25° C and another of 9° C. Although there seems to be strong evidence of a third group at 15° C, definite selections from these peaks have not been made.

An important feature throughout these experiments has been the striking similarity of the preferred temperature curves with respect to both the number and position of the various groups. In almost all the curves shown, representing over 8900 observations, the same number of groups were present, each congregating in their respective temperature zones at the same point along the gradient. Although only two of these have been isolated by selection, it would seem that the differences between them are clearly of genetic origin. The basis of such genetic differences as shown by the preferred temperature groups has not been investigated. It seems likely that by appropriate crosses a study of the hereditary mechanism involved may clarify the underlying relationship between the different groups.

The results of the present study, although of particular significance in connexion with the control of the European spruce sawfly in Canada, present points of general interest in biological control. A brief discussion may be made of the implications of these results on general questions of entomophagous parasite behaviour.

When parasites are reared in the laboratory, the ovipositing females are usually confined to the immediate vicinity of the host; *M. fuscipennis* Zett., for example, is placed in a small vial with the cocoon of the spruce sawfly. If an introduced parasite is to become established in the field, it must naturally frequent the host habitat. It is perhaps because they fail to do this that certain parasites extremely easy to breed in confinement have completely failed to breed in a new environment, even on their original host.

It is now known that some species will attack the host, only in certain limited parts of its range. Clark and Bradley (1939) have shown that *Chelonus annulipes* Wesm., a parasite of the European corn-borer (*Pyrausta nubilalis* Hubn.), is found only in certain restricted ecological habitats;

and unless the adults are liberated in or near one of these, colonization is not successful. *Microbracon brevicornis* Wesm., another parasite of *Pyrausta nubilalis* Hubn., is occasionally found attacking it in various parts of Europe but is of no importance except in Hungary. In Canada, large numbers of this parasite were bred and liberated; but it has never become established. It attacks the corn-borer in plants enclosed in cages even when the mesh is large enough to allow the escape of the parasite but apparently does not parasitize borers in plants in open fields.

The investigations of Parker (1918) and of Thorpe and Caudle (1939) indicate that in certain cases the adult parasites frequent different environments at different times and that their behaviour may be correlated with ovarian development; the female is attracted to the host habitat when she is ready to oviposit.

The problem of how parasites find their hosts in nature has not received much attention. Some authors have assumed that they locate them by random movement. In the case of *Microplectron fuscipennis* Zett., Ullyett (1936) states that 'The initial discovery of a host depends mainly on a chance encounter during the traverse of the particular area in which the parasite occurs'. This would lead one to conclude that the parasite moves at random until it is within a certain radius of the host, the radius being about 5–6 mm. under laboratory conditions. It then *perceives* the host and goes straight to it.

But it must be observed that this is merely the way the parasite behaves in a Petri dish, which in no way resembles the natural environment. As Thompson (1939) has recently pointed out, it is very unlikely that parasites find their hosts in nature by random movements. The evidence indicates rather that they are led to it by successive steps, being attracted first to the plants on which the host is found, then to the damaged areas and finally to the host itself. It thus appears that for the initial orientation of the parasite with respect to the host, the characteristics of the habitat may be of greater importance than those of the host itself.

The attraction exerted on the parasite by the host habitat probably depends on several factors, but it seems certain that among these temperature often plays an important part. The European spruce sawfly habitually spins its cocoon in the moss on the forest floor. The temperature in the moss is considerably lower than that of the air above it, and if it is distinctly below the preferendum for *Microplectron*, the parasite may not attack the host.

The experiments described in this paper show that the ovipositing females of *Microplectron* exhibit marked preferences for certain temperatures, and that the range of preferred temperatures of laboratory-reared specimens is rather limited. It is difficult to decide, from a priori considerations, whether the material reared in the laboratory by the routine methods adopted is really adapted to field conditions in the infested areas, because we have no way of estimating their ultimate effect. However, if we assume that an adaptive or selective process has occurred in Nature, the results of this should eventually manifest themselves in the field population.

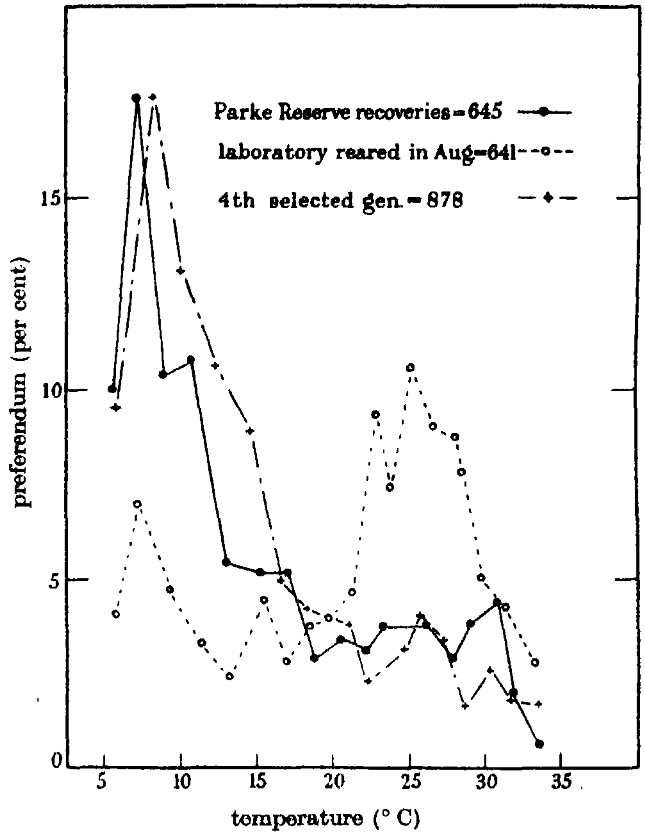

FIGURE 5. Preferred temperature curves of 'wild', normal laboratory, and selected stocks. The wild stock was secured from recovery collections made in August 1940, at Parke Reserve, Quebec.

Tests of the preferendum of material collected in the Gaspe peninsula in areas where the parasites had been established for some years, and thus had gone through a great number of generations under these conditions, were therefore made. In figure 5 curves are given showing the preferendum of adults recovered from Parke Reserve, Quebec (an area having a low mean temperature), females of the general laboratory line reared in August and fourth generation selections for low-temperature preference. It is evident that the preferendum of the 'wild' adults differs markedly from those reared in the laboratory. In the wild stock, 55 % of the individuals preferred temperatures below 15° C, as compared with 22 % in the laboratory-reared stock. On the other hand, the curve of the strain selected for low-temperature preference closely resembles that of the field material which suggests that natural selection, or an adaptive process in Nature, has produced the same results as were obtained by artificial selection in the laboratory.

The fact that the laboratory-reared material happened to have temperature preferences which are apparently ill-adapted to climates may explain the wide divergence in the results obtained from the liberation of *M. fuscipennis* Zett. in various parts of the infested area. In the heavily wooded, cooler sections of the Gaspe, more than 2–4 % parasitism has rarely been reported. On the other hand, liberations in southern New Brunswick, and the warmer sections of the Matapedia valley and the Upper St Lawrence, though made from the same stock, have resulted in some cases in a parasitism of 50–60 %, which has been attained in a much shorter period of time than the low parasitism of the cool regions (Baird 1939).

For conclusive proof of this theory, further field studies are required. Nevertheless, it does seem, from the investigations here recorded, that the laboratory-reared stock available in the initial stages of this work was not particularly well suited to the control of the European spruce sawfly in the Gaspe. Failure to find the host, produced by the unsuitable preferendum, might easily eliminate many small colonies and greatly retard the development of the parasite population.

On the other hand, the breeding of strains with specific preferenda, for liberation in particular sections of the area infested by a pest, or differing from those from which the parasite was originally obtained, might be distinctly advantageous. Effective establishment and rapid increase might thus be ensured, the cost of the work reduced and its practical value increased.

Finally, it seems probable that the methods utilized in this investigation, which has been restricted to the temperature preferences of the parasite, could be applied to other characteristics involved in the relations between parasite and host. The work of Thorpe and others has shown that the host preferences of parasites, dependent, apparently, on their reactions to various chemical emanations, are relatively plastic. Further analysis, combined with constructive breeding work on Mendelian lines, might allow us to duplicate in regard to parasites the results obtained with domestic animals and provide material far better adapted to the needs of the economic entomologist than that which Nature provides.

The progress of these studies was greatly facilitated by the availability of equipment at both the University of Toronto and the Dominion Parasite Laboratory, Belleville. The advice and assistance given by the staff of the

Department of Zoology, University of Toronto, more particularly Professor J. W. MacArthur, under whose direction the initial phases of the investigation were undertaken, are gratefully acknowledged. Many thanks are due to Mr A. B. Baird and the staff of the Dominion Parasite Laboratory for generous help. The writer wishes also to acknowledge indebtedness to Dr W. R. Thompson, F.R.S., of the Imperial Parasite Service, for valuable criticism and advice in the preparation of the manuscript.

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Theory of porometers used in the study of stomatal movements in leaves

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The mathematical theory of porometers, as used by Gregory and his collaborators for the study of stomatal movements in leaves, is given for viscous and diffusive flow of gases. It is shown that under certain conditions the theory applies to both kinds of gas movements; a correction is included for those cases in which the basic assumptions for diffusion are not realized. From determinations of the total leaf resistance to gaseous flow the conductance of the stomata can be found if the mesophyll resistance is known. The importance of this quantity is discussed and demonstrated, methods of measuring it are suggested, and possible checks upon the reliability of the result are described.

As an illustration, the theory is applied to some hitherto unpublished data on two pelargonium leaves; the treatment gives results that are internally consistent, and deduced rates of assimilation are found to be of the same order of magnitude as those observed in practice.

Progress in the study of stomatal movements in leaves has been considerably facilitated by the use of porometers. Accounts of the development of the instruments will be found in the papers cited below, and no more than a brief description of the principles involved is needed here. Two types are considered:

Resistance porometer (Gregory & Pearse 1934). A small cylindrical cup attached by means of a washer to one surface of the leaf is connected through a standard capillary resistance to a constant suction aspirator. Air is drawn through the leaf and standard capillary in series, and the pressure drop across each is measured; the ratio of the two pressure drops gives the ratio of the 'resistances' to viscous flow, so that from the known standard the equivalent capillary resistance of the leaf system can be calculated.

Diffusion porometer (Gregory & Armstrong 1936). The cylindrical cup is kept filled with pure hydrogen at 1 atm. pressure by flushing with a rapid stream of the gas. A differential diffusive flow of hydrogen and air through the leaf results, and this flow is measured in terms of the current needed to produce hydrogen electrolytically at a rate fast enough to keep the pressure in the cup constant. This current is a measure of the diffusive conductivity of the leaf system.

With both porometers measurement is made of a leaf resistance to fluid flow that arises from several sources. The purpose of this account is to separate the various factors and to interpret them, as far as possible, in absolute units. These factors are as follows:

(*a*) The resistance due to individual stomata on both leaf surfaces; on the lower surface three groups need consideration—inside and outside the cup and those effectively sealed by the thickness of the washer attaching the cup (see figure 1).

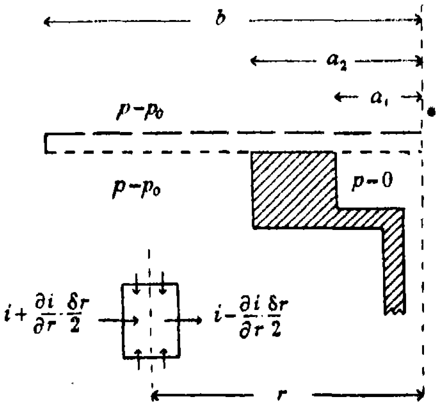
(*b*) The resistance to flow inside the leaf; two directions should be considered—parallel and normal to the leaf surface. In the analysis the latter is neglected, as the thickness of the leaf is very small in comparison with other dimensions. The mesophyll resistance is therefore considered as a superficial resistance parallel to the leaf surface.

Of these factors, set (*a*) is the one for which absolute data are required so that measures of stomatal aperture can be obtained which may be correlated with measured assimilation rates. In the second set, (*b*), originates much of the uncertainty as to the utility of porometer readings, and although Knight's double-cup method (1916) afforded in principle a method of measuring mesophyll resistance, yet hitherto hardly more than qualitative estimates have been made of the magnitude of this factor and therefore of the accuracy with which it should be measured. An approximate treatment of viscous flow by Newton (1936) failed to take into account the finite thickness of the washer of the porometer cup and also neglected lateral flow in the leaf tissue in the part under the cup.

The basic assumption for viscous flow is that a total pressure difference across the leaf causes a flow of air proportional to the pressure difference, i.e. that the 'resistance' of the leaf is independent of the pressure drop. The total pressure difference is also assumed so small that changes in density are negligible, a condition usually realized in practice. For diffusive flow the total pressure is assumed to be everywhere constant, and the relative flow of the two gases is determined by the existence of a partial pressure gradient. As long as this gradient is small, the flow of the two gases is equal and opposite, and proportional to the partial pressure difference causing diffusion. Thus, formally, the analysis for viscous and diffusive flow will be the same, provided that the pressure differences (total or partial) are small. These conditions are realized in practice for the viscous flow, but not so for diffusive flow, for here the partial pressure difference is 1 atm. The analysis is therefore developed as a problem in viscous flow, and is followed by a section in which the nature of the correction needed to adapt it to diffusive flow under non-ideal conditions is considered.

THEORY OF VISCOUS FLOW WITH CIRCULAR POROMETER CUP

The leaf, or part of the leaf, is assumed to be circular and bounded by an impervious wall around the circumference. Stomatal perforations in each of the surfaces permit the flow of fluids through the epidermis, and it is assumed that mean sizes of the two sets are equal, and that mutual interference between the stream-lines of flow through neighbouring stomata is either non-existent or affects both sets equally; hence the ratio of the conductivities through the two surfaces is given by the ratio of the numbers per unit area. The porometer cup is supposed applied symmetrically about the centre of the lower epidermis. The leaf is supposed so thin that there is no resistance to flow across the thickness, but there is resistance to flow within the leaf parallel to the plane of the leaf. It is assumed that pressure gradients are everywhere small so that changes in density can be neglected.



b
a_2
a_1
$p-p_0$
$p-p_0$
$p-0$
$i+\frac{\partial i}{\partial r}\frac{\delta r}{2}$
$i-\frac{\partial i}{\partial r}\frac{\delta r}{2}$
r

FIGURE 1. Idealized half-section of leaf with circular porometer cup attached. b, a_2 and a_1 are to scale (cf. figure 2).

If p_0 is the steady pressure drop between the outer air and that inside the cup, producing a radial flow into the cup, i_0 c.c./sec., then the ratio of these quantities, p_0/i_0, is the measured resistance of the leaf system, R. This resistance is due to the stomata and to the mesophyll. For the former it is convenient to work in terms of the conductivity, s, such that a pressure difference of δp across A cm.^{2} of leaf surface produces a current $As\delta p$ c.c./sec.; s_1 and s_2 will represent the epidermal conductivities of lower and upper surfaces and will be in reciprocal R-units/cm.^{2}. The mesophyll resistance is a surface effect, and we define the mesophyll resistivity, m, as the resistance of a strip 1 cm. wide and 1 cm. long. Hence, for an annulus $2\pi r$ cm. in circumference and δr cm. across, the radial current produced by a pressure difference δp is $\delta p\, 2\pi r/m\,\delta r$ c.c./sec.

Let b, a_2 and a_1 be the radii of the disk of leaf, the outer edge of the cup and the inner edge of the cup respectively. Also, let i be the total radial current inside the leaf at radius r and let p be the pressure excess at r over that in the cup.

Flow outside the cup ($b > r > a_2$). The pressure difference causing flow is $p_0 - p$ acting inward across both surfaces. Consider an annular ring, mean radius r, width δr. In the steady state the amount flowing into the element is equal to the amount flowing out:

Amount leaving per sec. at inner wall $= i - \dfrac{\partial i}{\partial r}\dfrac{\delta r}{2}$.

Amount entering per sec. at outer wall $= i + \dfrac{\partial i}{\partial r}\dfrac{\delta r}{2}$.

Amount entering per sec. at upper surface $= 2\pi r\,\delta r(p_0 - p)\,s_2$.

Amount entering per sec. at lower surface $= 2\pi r\,\delta r(p_0 - p)\,s_1$.

Hence
$$\frac{\partial i}{\partial r} = -2\pi r(s_1+s_2)\,(p_0-p).$$

Also
$$i = \delta p \,.\, 2\pi r/m\,\delta r,$$

or
$$\frac{\partial i}{\partial r} = \frac{2\pi}{m}\left(\frac{dp}{dr} + r\frac{d^2p}{dr^2}\right).$$

Hence in the range $b > r > a_2$
$$r^2\frac{d^2p}{dr^2} + r\frac{dp}{dr} = -r^2m(s_1+s_2)\,(p_0-p).$$

Now put $\rho_1 = r\sqrt{\{m(s_1+s_2)\}} = r\beta_1$, say, and $\phi_1 = p_0 - p$. The equation becomes
$$\rho_1\frac{d^2\phi_1}{d\rho_1^2} + \frac{d\phi_1}{d\rho_1} - \rho_1\phi_1 = 0. \qquad (1)$$

Flow inside the cup ($a_1 > r > 0$). The pressure difference across the upper surface is again $p_0 - p$ acting inward, but that across the lower surface is now p, acting outward. Accordingly, in the steady state
$$\frac{\partial i}{\partial r} = -2\pi r s_2(p_0-p) + 2\pi r s_1 p.$$

Again
$$\frac{\partial i}{\partial r} = \frac{2\pi}{m}\left(\frac{dp}{dr} + r\frac{d^2p}{dr^2}\right),$$

and
$$r^2\frac{d^2p}{dr^2} + r\frac{dp}{dr} = -r^2m[s_2(p_0-p) - s_1p]$$
$$= -r^2m(s_1+s_2)\left[\frac{s_2p_0}{s_1+s_2} - p\right].$$

Again put $\rho_1 = r\sqrt{\{m(s_1+s_2)\}} = r\beta_1$, and $\phi_2 = (s_2p_0)/(s_1+s_2) - p$. The equation becomes, in the range $a_1 > r > 0$,
$$\rho_1\frac{d^2\phi_2}{d\rho_1^2} + \frac{d\phi_2}{d\rho_1} - \rho_1\phi_2 = 0. \qquad (2)$$

Flow across the thickness of the washer $(a_2 > r > a_1)$. The equation can be derived from either of the above by putting $s_1 = 0$. Whence, putting $r\sqrt{(ms_2)} = \rho_2 = r\beta_2$ say,

$$\rho_2 \frac{d^2\phi_1}{d\rho_2^2} + \frac{d\phi_1}{d\rho_2} - \rho_2\phi_1 = 0. \tag{3}$$

For each equation there is the same formal type of solution and the solutions can be written

$$b > r > a_2, \quad \phi_1 = AI_0(\rho_1) + BK_0(\rho_1),$$

$$a_1 > r > 0, \quad \phi_2 = CI_0(\rho_1) + DK_0(\rho_1),$$

$$a_2 > r > a_1, \quad \phi_1 = FI_0(\rho_2) + GK_0(\rho_2),$$

where $I_0(\rho)$ and $K_0(\rho)$ are Bessel functions* and A, B, C, D, F and G are constants to be determined from the boundary conditions. These boundary conditions are: (i) as $r \to 0$, p remains finite; (ii) at $r = b$, there is no flow across the boundary; (iii)–(vi) at $r = a_2$ and $r = a_1$, the values of p and dp/dr are continuous. Hence, in order,

$$D = 0, \tag{i}$$

$$O = AI_1(\beta_1 b) - BK_1(\beta_1 b), \tag{ii}$$

$$AI_0(\beta_1 a_2) + BK_0(\beta_1 a_2) = FI_0(\beta_2 a_2) + GK_0(\beta_2 a_2), \tag{iii}$$

$$\beta_1[AI_1(\beta_1 a_2) - BK_1(\beta_1 a_2)] = \beta_2[FI_1(\beta_2 a_2) - GK_1(\beta_2 a_2)], \tag{iv}$$

$$FI_0(\beta_2 a_1) + GK_0(\beta_2 a_1) = CI_0(\beta_1 a_1) + \frac{s_1 p_0}{s_1 + s_2}, \tag{v}$$

$$\beta_2[FI_1(\beta_2 a_1) - GK_1(\beta_2 a_1)] = \beta_1[CI_1(\beta_1 a_1)]. \tag{vi}$$

From (ii), (iii) and (iv)

$$\sqrt{\left(\frac{s_2}{s_1 + s_2}\right)} \left[\frac{K_1(\beta_1 b)\, I_0(\beta_1 a_2) + I_1(\beta_1 b)\, K_0(\beta_1 a_2)}{K_1(\beta_1 b)\, I_1(\beta_1 a_2) - I_1(\beta_1 b)\, K_1(\beta_1 a_2)}\right] = \frac{F/G \,.\, I_0(\beta_2 a_2) + K_0(\beta_2 a_2)}{F/G \,.\, I_1(\beta_2 a_2) - K_1(\beta_2 a_2)}. \tag{4}$$

From (v) and (vi):

$$\sqrt{\left(\frac{s_2}{s_1 + s_2}\right)} \left[\frac{CI_0(\beta_1 a_1) + \dfrac{s_1 p_0}{s_1 + s_2}}{CI_1(\beta_1 a_1)}\right] = \frac{F/G \,.\, I_0(\beta_2 a_1) + K_0(\beta_2 a_1)}{F/G \,.\, I_1(\beta_2 a_1) - K_1(\beta_2 a_1)}. \tag{5}$$

Thus for any assumed value of β_1, given the dimensional constants b, a_2 and a_1, and the ratio s_2/s_1 ($= \alpha$, say), the value of C can be found from the second of these equations, the first being used to evaluate F/G.

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* The tables employed in this part of the work are *B.A. Mathematical Tables, Bessel Functions*, Part 1 (Cambridge Univ. Press, 1937).

The total flow through the cup, $i_0 = p_0/R$, where R is the measured resistance. Therefore

$$\begin{aligned} i_0 = p_0/R &= \int_0^{a_1} p \,.\, 2\pi r s_1 dr \\ &= \frac{2\pi s_1}{m(s_1+s_2)} \int_0^{\beta_1 a_1} \left[\frac{s_2 p_0}{s_1+s_2} - C I_0(\rho_1)\right] \rho_1 d\rho_1 \\ &= \frac{2\pi s_1}{m(s_1+s_2)} \left[\frac{s_2 p_0}{s_1+s_2} \frac{\beta_1^2 a_1^2}{2} - C\beta_1 a_1 I_1(\beta_1 a_1)\right]. \qquad (6)^* \end{aligned}$$

To show the type of solution eventually obtained consider an ideal case. Put a_2 equal to a_1. Then the right-hand sides of equations (4) and (5) are identical, and equating the left-hand sides,†

$$C = \frac{s_1 p_0}{s_1+s_2} \frac{\beta_1 a_1}{I_1(\beta_1 b)} [I_1(\beta_1 a_1) K_1(\beta_1 b) - K_1(\beta_1 a_1) I_1(\beta_1 b)].$$

Hence

$$\frac{p_0}{R} = \frac{2\pi s_1}{m(s_1+s_2)^2} p_0 \beta_1^2 a_1^2 \left\{\frac{s_2}{2} - s_1 \frac{I_1(\beta_1 a_1)}{I_1(\beta_1 b)} [I_1(\beta_1 a_1) K_1(\beta_1 b) - K_1(\beta_1 a_1) I_1(\beta_1 b)]\right\},$$

i.e.
$$\frac{m}{R} = \frac{2\pi\beta_1^2 a_1^2}{(1+\alpha)^2} \left\{\frac{\alpha}{2} - \frac{I_1(\beta_1 a_1)}{I_1(\beta_1 b)} [I_1(\beta_1 a_1) K_1(\beta_1 b) - K_1(\beta_1 a_1) I_1(\beta_1 b)]\right\}.$$

Also
$$\beta_1^2 = m(s_1+s_2), \quad \text{i.e.} \quad ms_1 = \beta_1^2/(1+\alpha).$$

Thus, for any assumed value of β_1, values of m/R and ms_1 can be estimated.

Special case. For a hypostomatous leaf we have $s_2 = 0$ and hence $\alpha = 0$. When $\alpha = 0$ several terms in equations (4) and (5) become either zero or infinity and a separate treatment of this case is needed. Making use of the fact that for $a_2 > r > a_1$ there is no further change in i, so that

$$a_2 > r > a_1, \quad i_0 = \frac{2\pi}{m} r \frac{dp}{dr},$$

i.e.
$$\left.\frac{dp}{dr}\right|_{r=a_2} = \frac{mi_0}{2\pi} \frac{1}{a_2}; \quad \left.\frac{dp}{dr}\right|_{r=a_1} = \frac{mi_0}{2\pi} \frac{1}{a_1},$$

and
$$p_{a_2} - p_{a_1} = \frac{mi_0}{2\pi} \log_e \frac{a_2}{a_1}.$$

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*
$$\begin{aligned} x I_0(x) &= I_1(x) + x I_1'(x), \\ \therefore \int_0^x x I_0(x)\, dx &= \int_0^x I_1(x)\, dx + \int_0^x x I_1'(x)\, dx \\ &= I_0(x) + x I_1(x) - I_0(x) \\ &= x I_1(x). \end{aligned}$$

† Using the relation (McRobert, *Spherical harmonics*, p. 271)

$$K_1(x)\, I_0(x) + K_0(x)\, I_1(x) = 1/x.$$

We obtain

$$\frac{K_1(\beta_1 b)\,I_0(\beta_1 a_2)+I_1(\beta_1 b)\,K_0(\beta_1 a_2)}{K_1(\beta_1 b)\,I_1(\beta_1 a_2)-I_1(\beta_1 b)\,K_1(\beta_1 a_2)} = \frac{CI_0(\beta_1 a_1)+\left(p_0-\dfrac{mi_0}{2\pi}\log_e\dfrac{a_2}{a_1}\right)}{\dfrac{a_1}{a_2}CI_1(\beta_1 a_1)}$$

$$= M \text{ say.}$$

Then

$$C = \frac{p_0-\dfrac{mp_0}{2\pi R}\log_e\dfrac{a_2}{a_1}}{M\dfrac{a_1}{a_2}I_1(\beta_1 a_1)-I_0(\beta_1 a_1)},$$

leading to

$$\frac{2\pi R}{m} = -\frac{M\dfrac{a_1}{a_2}-\dfrac{I_0(\beta_1 a_1)}{I_1(\beta_1 a_1)}}{\beta_1 a_1}+\log_e\frac{a_2}{a_1}. \tag{7}$$

The curves of figure 2 have been derived in this way, using equations (4), (5) and (6) for $\alpha = 0{\cdot}04$ and $\alpha = 0{\cdot}10$, and using equation (7) for $\alpha = 0{\cdot}00$. The values of β_1 used are such that $\beta_1 a_1 = 0{\cdot}05$, $0{\cdot}20$, $0{\cdot}80$, $1{\cdot}60$, $3{\cdot}20$, $6{\cdot}40$, $12{\cdot}80$ and $20{\cdot}0$. Other constants used in figure 2 are $b = 2{\cdot}0$ cm., $a_2 = 0{\cdot}85$ cm., $a_1 = 0{\cdot}45$ cm. To obtain a value of s_1 for an observed R it is necessary to know m.

Limiting values. (i) *Small stomatal aperture.* By lengthy algebra, which cannot justifiably be reproduced here, it is found, for all small values of α, as $\beta_1 \to 0$, that

$$C \to -\frac{b^2-a_2^2}{b^2-a_2^2+a_1^2}\cdot\frac{s_1 p_0}{s_1+s_2},$$

i.e. at small values of ms_1 equation (6) reduces to

$$\frac{p_0}{R} = \pi a_1^2 s_1\left[\frac{s_2 p_0}{s_1+s_2}+\frac{s_1 p_0}{s_1+s_2}\cdot\frac{b^2-a_2^2}{b^2-a_2^2+a_1^2}\right].$$

Hence, when $b^2-a_2^2$ is large compared with a_1^2,

$$1/R = \pi a_1^2 s_1, \tag{8}$$

or all the resistance is due to the part under the cup.

(ii) *Large stomatal aperture.* As β_1 and $\beta_2 \to \infty$, β_2/β_1, and hence α, remaining constant and finite and not zero, the value of $CI_0(\beta_1 a_1)$ tends to

$$-\frac{s_1 p_0}{(s_1+s_2)\left\{1+\sqrt{\left(\dfrac{s_1+s_2}{s_2}\right)}\right\}},$$

i.e.

$$\frac{p_0}{R} \to \frac{2\pi s_1 p_0 \beta_1^2 a_1^2}{m(s_1+s_2)^2}\left[\frac{s_2}{2}+\frac{s_1}{\beta_1 a_1\left\{1+\sqrt{\left(\dfrac{s_1+s_2}{s_2}\right)}\right\}}\right]$$

$$\to \pi a_1^2 p_0\frac{s_1 s_2}{s_1+s_2}\left[1+\frac{2}{\alpha a_1 \beta_1\left\{1+\sqrt{\left(\dfrac{s_1+s_2}{s_2}\right)}\right\}}\right].$$

As $\beta_1 \to \infty$ the second term in the bracket slowly $\to 0$, the convergence being slower the smaller the value of α. In the limit

$$\frac{1}{R} = \pi a_1^2 \frac{s_1 s_2}{s_1 + s_2},$$

or,

$$R = \frac{1}{\pi a_1^2 s_1} + \frac{1}{\pi a_1^2 s_2}, \tag{9}$$

i.e. the resistance is wholly due to the resistances of the upper and lower parts of the leaf under the cup.

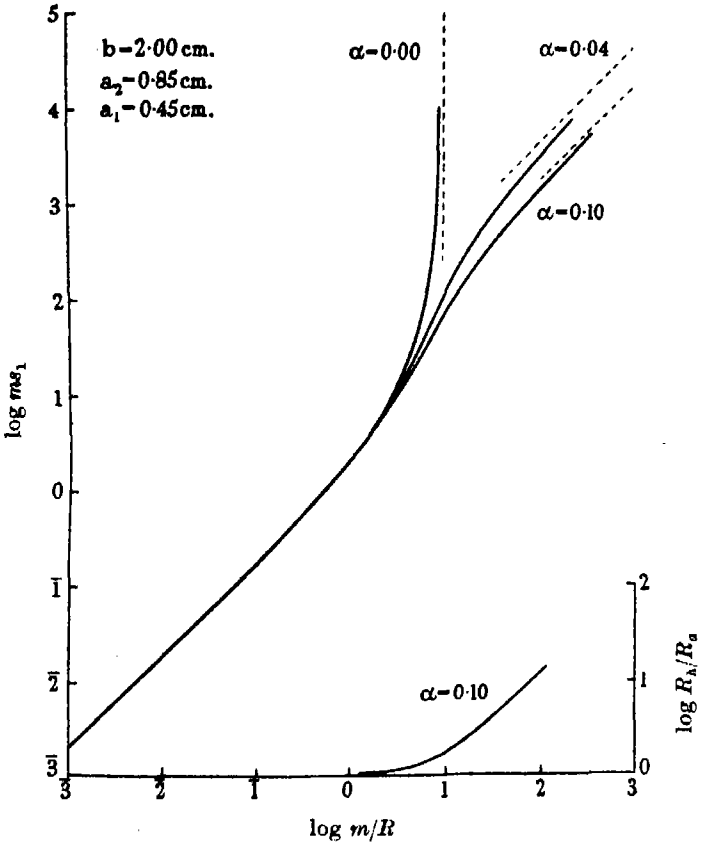

FIGURE 2. Curves for conversion of observed total leaf resistance (R) into stomatal conductivity per cm.^{2} (s_1) for various values of ratio of upper to lower surface conductivities (α).

(iii) *Large stomatal aperture* (hypostomatous leaf). When β_2 and hence α are zero, then as β_1 increases, $M \to -1$, and from equation (7)

$$\frac{2\pi R}{m} \to \frac{1}{\beta_1}\left(\frac{1}{a_2} + \frac{1}{a_1}\right) + \log_e \frac{a_2}{a_1},$$

i.e. in the limit, as $\beta_1 \to \infty$ $\qquad R \to \frac{m}{2\pi}\log_e\frac{a_2}{a_1}.$ (10)

The resistance is entirely due to the mesophyll under the washer. The upper asymptotes have been drawn in figure 2. That for $\alpha = 0$ is given by the equation immediately above. For $\alpha \neq 0$,

$$\frac{m}{R} = ms_1 \pi a_1^2 \frac{\alpha}{1+\alpha}$$

from equation (9), and the equation of the upper asymptote is

$$\log ms_1 = \log\frac{m}{R} - \log \pi a_1^2 + \log\frac{1+\alpha}{\alpha}.$$

For the lower asymptote, common to all small α, the equation for large b is

$$\log ms_1 = \log\frac{m}{R} - \log \pi a_1^2$$

from equation (8), i.e. the upper asymptote is parallel to the lower.

DETERMINATION OF MESOPHYLL RESISTANCE

To determine m let us suppose that the upper epidermis is closed ($s_2 = 0$) and everything kept constant except a_2 which is increased to a_3. With an obvious symbolism, M_2 becomes M_3, R_2 becomes R_3, and putting

$$I_0(\beta_1 a_1)/\beta_1 a_1 I_1(\beta_1 a_1) = \gamma,$$

we have from (7),

$$R_2 = -\frac{m}{2\pi}\left[\frac{M_2}{a_2\beta_1} - \gamma - \log_e\frac{a_2}{a_1}\right],$$

$$R_3 = -\frac{m}{2\pi}\left[\frac{M_3}{a_3\beta_1} - \gamma - \log_e\frac{a_3}{a_1}\right],$$

$$R_3 - R_2 = -\frac{m}{2\pi}\left[\frac{1}{\beta_1}\left(\frac{M_3}{a_3} - \frac{M_2}{a_2}\right) - \log_e\frac{a_3}{a_2}\right]. \tag{11}$$

Assume, as a first approximation, that the first term in the bracket is negligible: an approximate value of m, m_1 say, is $= [2\pi(R_3 - R_2)]/[\log_e a_3/a_2]$. Using this value of m_1 and the known value of R_2, $m_1 s_1$ ($= \beta_1^2$) can be read from the curve for $\alpha = 0$, i.e. values of $\beta_1 a_1$, $\beta_1 a_2$ and $\beta_1 a_3$ can be found. Thus values of M_3 and M_2 can be estimated, leading to a more exact estimate of m. Further discussion of the importance of m and an alternative method of measuring it will be found below (p. 427).

THEORY OF DIFFUSIVE FLOW

As indicated above (p. 417) the theory for viscous flow will also apply to diffusive flow under small partial pressure gradients. In the present section I shall follow Trautz & Müller (1935) in considering the treatment of the case in which the two gas flows are not equal, and although the extrapolation to 1 atm. partial pressure difference may be too large it will at least give a first approximation.

For small partial pressure gradients the coefficient of diffusion, D_0 say, of two gases is defined as the rate of flow of gas per unit area per unit density gradient. If ρ_1, ρ_2 be the densities of the gases (g./c.c.), u_1 and u_2 be the mass velocities in the direction of x increasing (cm./sec.), then

$$\rho_1 u_1 = -D_0 \frac{\partial \rho_1}{\partial x}, \quad \text{or} \quad \rho_2 u_2 = -D_0 \frac{\partial \rho_2}{\partial x}.$$

If p_1 and p_2 be the corresponding partial pressures such that $p_1 = k_1 \rho_1$, $p_2 = k_2 \rho_2$ and $p_1 + p_2 = P$ = constant, it can be shown that $D_0 = k_1 k_2 / CP$, where C is a constant in the general equation of motion

$$\frac{dp_1}{dx} + C\rho_1 \rho_2 (u_1 - u_2) = 0.$$

Normally, $\rho_1 u_1 = \rho_2 u_2$, i.e. the masses of the two gases crossing any given plane are equal. Next consider the case in which they are not. Assume that the partial pressures are developed at two parallel planes of area A sq. cm. and effectively l cm. of air apart.

$$k_1 \frac{d\rho_1}{dx} + C\rho_1 \rho_2 (u_1 - u_2) = 0,$$

i.e.

$$\frac{d\rho_1}{dx} = \frac{k_2}{D_0 P} \rho_1 \rho_2 (u_2 - u_1).$$

Putting $\rho_1 u_1 = m_1$, $\rho_2 u_2 = m_2$, where m is in g./sec./cm.^{2}. Then

$$\frac{d\rho_1}{dx} = \frac{k_2}{D_0 P} (\rho_1 m_2 - \rho_2 m_1)$$

$$= \frac{k_2}{D_0 P k_1} [m_2 P - \rho_2 (m_2 k_2 + m_1 k_1)]$$

$$= -\frac{d\rho_2}{dx} \frac{k_2}{k_1},$$

i.e.

$$-\frac{dx}{D_0 P} = \frac{d\rho_2}{m_2 P - \rho_2 (m_2 k_2 + m_1 k_1)},$$

or, integrating from 0 to l,

$$\frac{l}{D_0 P}(m_2 k_2 + m_1 k_1) = \log_e \Big[m_2 P - \rho_2(m_2 k_2 + m_1 k_1) \Big]_0^l$$

$$= \log_e \Big[m_2 \rho_1 - m_1 \rho_2 \Big]_0^l.$$

Suppose ρ_1 refers to hydrogen and that it is at pressure P at $x = 0$, while ρ_2 refers to air (at P at $x = l$), both measured at $T°$ K, the temperature of the experiment. Let ρ_H and ρ_A be the densities of H_2 and air at P, 273°. Then at $x = 0$; $\rho_1 = \rho_H . 273/T$, $\rho_2 = 0$. At $x = l$; $\rho_1 = 0$, $\rho_2 = \rho_A . 273/T$. Also let V_1 be the volume of hydrogen flowing $0 \to l$ measured at P, 273, and V_2 be the volume flow of air $l \to 0$, also measured at P, 273. Then $m_2 = -(V_2 \rho_A)/A$, $m_1 = (V_1 \rho_H)/A$. We obtain

$$\frac{l}{AD_0 P}(-V_2 \rho_A k_2 + V_1 \rho_H k_1) = \log_e \frac{-m_1 \rho_A}{m_2 \rho_H},$$

where $P . T/273 = k_1 \rho_H = k_2 \rho_A$, i.e.

$$\frac{l}{AD_0}(V_1 - V_2)\frac{T}{273} = \log_e \frac{V_1}{V_2},$$

where $V_1 - V_2$ is the differential flow of hydrogen and air. This is the measured flow, V c.c./sec. To evaluate V_1/V_2, assume that Graham's law of diffusion holds, i.e. that

$$V_1/V_2 = \sqrt{(\rho_A/\rho_H)} = 3{\cdot}8.$$

Hence

$$\frac{l}{AD_0} = \frac{\log_e 3{\cdot}8}{V}\frac{273}{T} = \frac{1{\cdot}335}{V}\frac{273}{T}.$$

For small partial pressure gradients the diffusion equation for the same system can be written

$$\frac{dq}{dt} = D_0 A \frac{\delta c}{l},$$

where dq/dt is the equal and opposite volume flow rate of the two gases and δc is the partial concentration difference (c.c./c.c.). Defining diffusive impedance, (Z), as the ratio of concentration difference to flow produced,

$$Z = \frac{l}{AD_0} = \frac{1{\cdot}335}{V}\frac{273}{T}. \tag{12}$$

This value gives the impedance of the system under the ideal conditions in which the theory for viscous flow can be applied to diffusive flow. Thus a double cup experiment, if practicable, could be used to obtain the mesophyll impedance, Z_m, and curves such as figure 2 used to obtain the stomatal conductivity, $1/Z_s$.

General considerations. (i) *Units.* As defined, the viscous resistance and diffusive impedance include terms characteristic of the moving fluid (coefficient of viscosity or diffusion) and terms depending on the geometry of the leaf. As the chief interest is in the latter, it is desirable to eliminate the former. The interrelations of R, m and s_1 (or of Z, Z_m and $1/Z_s$), are such that any unit of resistance (or impedance) can be used since m is measured in units of R (equation (11)), and the derived value of s_1 is automatically obtained in reciprocal R-units per square cm. If, therefore, R is estimated in capillary units, the value of s_1 obtained will be $\Sigma_n r^4/l_V$, where n is the number of stomata/cm.^{2}, and r and l_V are the radius and length of the cylindrical tube that would have the same viscous resistance as an average stoma. The viscous variables may be conveniently re-symbolized thus: R_V, m_V and s_V.

In a similar way the dimensional term (R_D say) can be separated in the diffusive impedance. Then

$$R_D = \left[\frac{l}{A}\right] = \frac{1\cdot335}{V}\,\frac{273}{T}\,D_0,$$

or, since $D_0 = 0\cdot64(T/273)^2$,

$$R_D = \left[\frac{l}{A}\right] = \frac{0\cdot854}{V}\,\frac{T}{273}. \tag{13}$$

The derived value of s_D will therefore represent $\Sigma_n A/l_D$ where A and l_D are the effective area and length of a prism that would have the same conductance as an average stoma. Note: it is not postulated here that $l_V \equiv l_D$, or that $A \equiv \pi r^2$.

(ii) *Resistance to flow inside the leaf.* Neglect of the resistance across the leaf has been regarded as reasonable because of the extreme thinness of the system. If m is precisely known a check on this assumption is possible by measuring first the value of R_a under amphistomatous conditions and then sealing the upper surface to obtain R_h under hypostomatous conditions. If the resistance across the leaf *is* negligible the two values of ms_1 ought to agree. As will be seen below, the range of m/R in which this experiment could be usefully employed is one in which the accuracy of m is important and it would be more effective to use the experiment to determine m, assuming the resistance across the leaf is negligible. From the theoretical curves a derived curve of R_h/R_a against m/R_a can be plotted (see figure 2); from the experiment a value of R_h/R_a is found, and hence a value of m/R_a. This, with the known value of R_a, leads to a value of m.

The mesophyll resistance to viscous flow depends upon the air-filled pore-space in the leaf tissue and also on the frequency distribution of pore-sizes; hence it is not easily predictable from first principles. The corresponding diffusive resistance depends only on the total air-filled pore-space,

S, and if this can be determined from leaf-sections, an estimate of m_D can be made as follows. In the range $0 < S < 0{\cdot}6$ the effective coefficient of diffusion through a porous solid is

$$D = 0{\cdot}66 D_0 S \quad \text{(Penman 1940).}$$

Hence for diffusive flow in a square of leaf, thickness t cm., parallel to the plane of the leaf, we have

$$Z_m = A/lD = 1/0{\cdot}66 D_0 St,$$

i.e.

$$m_D = 1/0{\cdot}66 St. \tag{14}$$

The measurements of the air-filled pore-space and of the leaf thickness (bottom of palisade to lower epidermis) are not easy to carry out with certainty, and this method of estimating m_D is offered for use only where no experimental determinations have been made.

(iii) *Importance of mesophyll resistance.* Since at any point on the curves of figure 2,

$$\frac{\Delta \log m s_1}{\Delta \log m/R} = \mu \text{ say,}$$

it follows that

$$\frac{1}{m s_1}[s_1 \Delta m + m \Delta s_1] = \mu \frac{\Delta m}{m},$$

or,

$$\Delta s_1/s_1 = (\mu - 1)\Delta m/m.$$

Thus when the slope is unity, or near it, the value of m is not very critical, but as μ approaches 2 the accuracy of m must be equal to the desired accuracy in s_1. For $\mu > 2$ the accuracy of m becomes even more important, and results from such parts of the curves should be used with caution.

ASSIMILATION RATES

The rate at which carbon dioxide will be removed by the leaf from the outside atmosphere will depend upon the distance the gas must penetrate into the leaf before being completely assimilated. The maximum rate for any given stomatal aperture would be obtained if assimilation were complete just inside the epidermis. Assuming this condition to be satisfied, we may write down an expression for the assimilation rate

$$V_{CO_2} = D_{CO_2} s_D \delta c \,.\, 273/T \text{ c.c./cm.}^2\text{/sec., measured at } P, 273,$$

where δc is the concentration difference across the epidermis ($\delta c \fallingdotseq 3 \times 10^{-4}$ for average atmospheric air). Substituting the known value of D for carbon dioxide-air, we obtain

$$V_{CO_2} = 0{\cdot}139(T/273)^2 s_D \delta c \,.\, 273/T \quad \text{c.c./cm.}^2\text{/sec.,}$$

or

$$M_{CO_2} = 10{\cdot}4 s_D \delta c \times 10^4 \text{ mg./dm.}^2\text{/hr.} \quad \text{for} \quad T = 290^\circ \text{K}. \tag{15}$$

EXPERIMENTAL DATA

Although it is hoped that the preceding analysis will be useful wherever porometers are used, the immediate stimulus came from the need of interpreting the results of flow experiments by Mr O. V. S. Heath. Detailed accounts of apparatus and technique (Heath 1939), and of the viscous flow porometer (Heath 1941), have appeared or are in press, and a future publication from the same author will discuss the diffusive flow experiments and the relation between viscous and diffusive flow results. The present discussion will be restricted to an indication of the effect of treating the experimental data in the way outlined above; in the future publication the experimental accuracy and the biological significance of agreements and differences will be discussed for all the experimental data.

Measurements of viscous resistance and diffusive flow were made on leaves of *Pelargonium zonale* on 16 November 1939 ($\alpha = 0{\cdot}10$) and 28 November 1939 ($\alpha = 0{\cdot}00$), the porometer cups having the dimensions used in deriving figure 2. Heath (1941) gives the data for double cup experiments from which m_V was found to be 4·85 Gregory & Pearse units* for other pelargonium leaves. It is assumed that this value will hold for the leaves considered here and also that there is a common value of m_D yet to be determined. The experiments consisted in following the changes in diffusive and viscous conductivity as the stomata opened under a 100 W lamp and closed in the dark. Between each pair of diffusive readings several viscous observations were made, and by interpolating, the value of the viscous resistance at the time of making a diffusive reading was estimated. Using these values of R_V, $\log m_V/R_V$ and hence $\log m_V \cdot s_V$ were estimated and graphed (figure 3).

TABLE 1. DETERMINATION OF m_D

$\log m_V s_V$	$\log R_{Dh}/R_{Da}$	$\log m_D/R_{Da}$	$\log R_{Da}$	$\log m_D$
0·800	0·210	0·965	1·380	2·345
1·200	0·275	1·065	1·270	2·335
1·600	0·340	1·160	1·185	2·345
2·000	0·380	1·210	1·135	2·345
2·200	0·400	1·235	1·120	2·355

Mean $\log m_D = 2{\cdot}345$. $m_D = 221$ cm.$^{-1}$.

To determine m_D the method outlined on p. 427 has been used. To each $m_V s_V$ of figure 3 there corresponds a value of R_{Dh} and of R_{Da}. From the experimental data a plot of $\log R_{Dh}$ and $\log R_{Da}$ against $\log m_V s_V$ was made and smooth curves drawn through the points. At five values of $\log m_V s_V$ on the opening curves the values of $\log R_{Dh}/R_{Da}$ were read off, and from the derived curve of figure 2 were found the corresponding values of $\log m_D/R_{Da}$. Hence five values of m_D were found.

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* One Gregory & Pearse unit $\doteqdot 3{\cdot}77 \times 10^{8}$ cm.$^{-3}$ (Heath 1939).

The values of R_D were estimated from equation (13). The experimental quantity measured is C, the current in milliamperes, and using the known electro-chemical equivalent of hydrogen it follows that

$$V = C \times 1{\cdot}05 \times 10^{-8} \times 22{,}400/2 \text{ c.c./sec.}$$

Hence, at $T = 290^\circ$ K, $\log R_D = 3{\cdot}887 - \log C$, and for $\log m_D = 2{\cdot}345$,

$$\log m_D/R_D = \log C - 1{\cdot}542. \tag{13a}$$

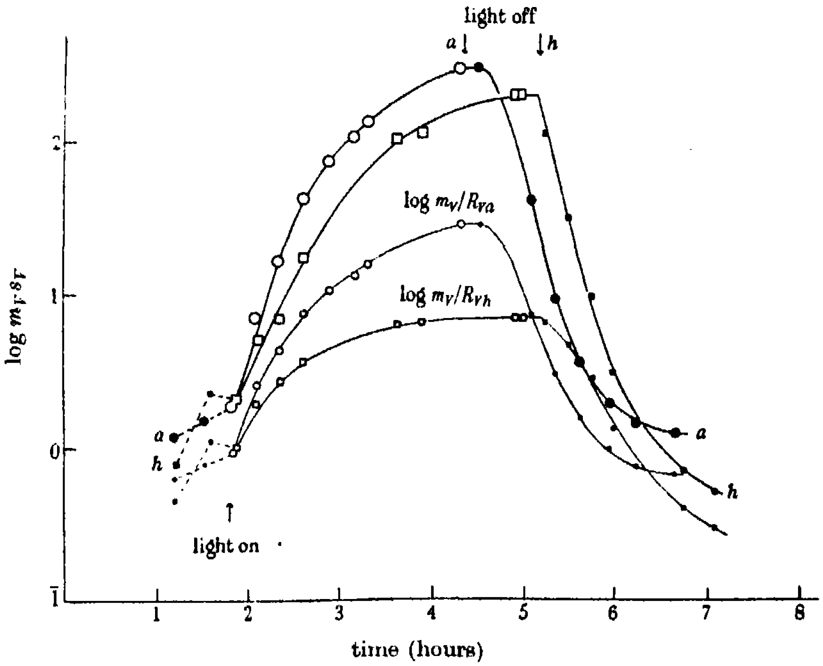

FIGURE 3. Time curves for *viscous* flow through pelargonium leaves, showing changes in stomatal conductivity (s_V) and leaf conductivity ($1/R_V$) for an amphistomatous leaf (a; $\alpha = 0{\cdot}10$) and a hypostomatous leaf (h; $\alpha = 0{\cdot}00$). Open circles and squares represent the period of irradiation. $m_V = 4{\cdot}85 \times 3{\cdot}77 \times 10^8$ cm.$^{-3}$.

Figure 4 shows how $\log m_D s_D$ increases during the light period and how it decreases in the following dark period. In each of the figures, the values of $\log m/R$ are plotted. Note that the scale for $\log m_D s_D$ is twice that for $\log m_V s_V$. Several points of interest emerge.

(i) Although the curves for $\log m/R_h$ and $\log m/R_a$ differ considerably, the derived curves of $\log ms$ are very similar in phase and amplitude. Differences are to be expected because all leaves are not alike and their individual responses to light will presumably be conditioned by their previous history.

(ii) The values of $m_D s_D$ appear to reach a maximum before the values of $m_V s_V$.

(iii) The apparent amplitudes for the two figures are about equal, i.e. the range of $\log m_V s_V$ is about twice that for $\log m_D s_D$.

(iv) There is a hysteresis between $\log m_D s_D$ and $\log m_V s_V$. For a constant value of the latter, the value of the former is less in the closing stage than in the opening stage; for $\log m_V s_V = 1{\cdot}22$ we have

		$\alpha = 0{\cdot}00$	$\alpha = 0{\cdot}10$
$\log m_D s_D$:	Opening	1·94	1·95
	Closing	1·60	1·87

Note that the width of the hysteresis loop is 0·34 for the hypostomatous leaf and 0·08 for the amphistomatous leaf.

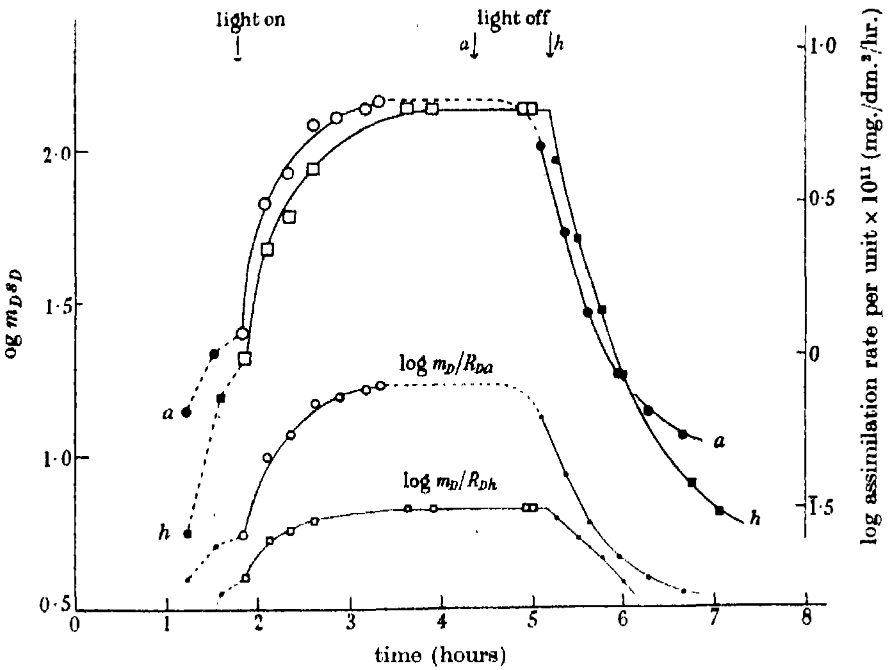

FIGURE 4. Time curves for *diffusive* flow through pelargonium leaves, showing changes in stomatal conductivity (s_D) and leaf conductivity ($1/R_D$) for an amphistomatous leaf (a; $\alpha = 0{\cdot}10$) and a hypostomatous leaf (h; $\alpha = 0{\cdot}00$). Open circles and squares represent the period of irradiation. $m_D = 221$ cm.$^{-1}$. δc is the CO_2 concentration difference (c.c./c.c.) across the epidermis.

Some of these features ((ii) and (iv)) were already apparent in the untreated experimental results; although the analysis may have modified their form and magnitude, it has not created them. Discussion of their possible biological implications is withheld until all the available data are analysed; the remainder of this account will consider some analytical aspects of the curves, chiefly from the point of view of accuracy in handling diffusion data.

The value of 2·345 for $\log m_D$ was obtained from the opening curves of R_{Dh} and R_{Da}. Using the same method a second mean value can be obtained from the closing curves and is found to be $\log m_D = 2{\cdot}43$ with a range of 2·37–2·46, the higher mean being an effect of hysteresis and the greater spread being due to the different shapes of the hysteresis curves for the two leaves. This value of m_D would give curves of $\log m_D s_D$ with higher maxima, especially for the hypostomatous leaf. Normally one would take as the effective value a mean from opening and closing curves, weighted according to the reliability of the experimental data, but in the present illustration only the opening data have been used because of their greater internal consistency. A further value of m_D can be obtained by making another assumption. For the hypostomatous leaf the flat-topped curve by itself might mean that we have reached the limiting value of m/R to which equation (10) applies. Using the nominal values of a_2 and a_1 we find $\log m_D = 2{\cdot}51$. There are two reasons for rejecting this value as too large. In the first place the gelatine washer, thickness $a_2 - a_1$, would spread slightly on being pressed against the leaf, decreasing a_1 and increasing a_2 and, more important, the grease used to ensure a gas-tight joint would spread a little on either side of the washer in making the contact and hence a_2/a_1 would be effectively larger than its nominal value. The second reason for suggesting that $\log m_D < 2{\cdot}51$ is that a flat-topped curve is also obtained for the amphistomatous leaf. This result would not be obtained while the diffusive conductivity was still increasing, and it seems that the explanation of the flat maxima is that within the limits of experimental accuracy the diffusive conductivity has reached its maximum value, and not that the value of m_D/R_{Dh} has reached its asymptotic limit. To these experimental methods of measuring m_D can be added the theoretical deduction of m_D indicated in equation (14) above. For the pelargonium leaves used, Heath (1941) estimates the mean pore-space, S, as about 30 %, and the thickness of the leaf, t, as 100μ, or 136μ if the palisade is included. Assuming that gaseous flow is confined to the spongy mesophyll tissue, we have

$$m_D = 1/0{\cdot}66St$$

$$\fallingdotseq 500 \quad \text{for} \quad t = 0{\cdot}01\ \text{cm.},\ S = 0{\cdot}30.$$

The constant in this equation, 1/0·66, is a measure of the meandering of the diffusion paths between cells. If these paths are nearly straight the constant approaches unity, and as a lower limit for the same S and t, we have $m_D \geqslant 333$. Hence one would expect $\log m_D$ to lie between 2·52 and 2·70; there would be a decrease in both limits if the values of S and t for the leaf concerned were greater than the mean values used in the calculation.

The values of $\log m_D$ obtained in these various ways are all of the same order, but the values of $\log m_D/R_{Dh}$ are so near the asymptotic limit that a small error in m_D is extremely important, much more important than a corresponding error in m_V, because, although the maximum values of both m_D/R_{Dh} and m_V/R_{Vh} bring us into the critical region, many more of the diffusive readings are near this region than are the corresponding viscous readings. The double cup method of measuring mesophyll resistance has hitherto been possible only for m_V, but if the results of diffusive flow experiments are to be fully and confidently interpreted, the admittedly difficult task of extending the method to measuring m_D should be incorporated in experimental technique. Mr Heath is considering this matter.

Two internal qualitative checks on the suitability of the value used may be applied. (*a*) If one can assume that the stomata of the same kind of leaf will always open to about the same extent for the same photo-stimulus, then agreement in maximum values of $m_D s_D$ (cf. figure 4) will indicate a reasonable estimate of m_D. (*b*) Where assimilation rates are measured and correlated with s_D it might be legitimate to assume from other evidence that over part of the range the rate is proportional to s_D, and the suitability of the value of m_D could be checked by the agreement between observed and theoretical values of assimilation rate over this range. These theoretical values will be obtained from equation (15); for unit value of $\delta c \times 10^4$ the scale of theoretical assimilation rates has been given on the right-hand side of figure 4. As a composite illustration, in table 2 are given the theoretical maximum assimilation rates for the two leaves, calculated for the various values of $\log m_D$ that have been considered above. The value of the mean concentration difference is taken as $\delta c = 2 \times 10^{-4}$, known to be reasonable for the experiments concerned, and the two points to be looked for are the agreement of the values for the two leaves, first with each other and second with the known maximum rate (*c.* 10 mg./dm.^{2}/hr.); as the quoted values of the observed maximum and of δc are only approximate the second test is to be applied to the order of magnitude only.

TABLE 2. THEORETICAL ASSIMILATION RATES (MG./DM.^{2}/HR.)

	$\log m_D$	2·345	2·43	2·51	2·70
Assimilation rate	$\alpha = 0{\cdot}10$	13·5	14·7	15·8	24·0
	$\alpha = 0{\cdot}00$	12·6	61·5	∞	?

The first test indicates that $\log m_D$ is either 2·345 or very near this value; the second, if we may disregard the hypostomatous leaf data, indicates that any of the three lower values could be taken as reasonable, and even the fourth is far from being absurd. The combined evidence of the table is in favour of the lowest value.

The importance of obtaining a precise value of m_D has probably received sufficient emphasis already. If more were needed it could be found in table 2 where the effect of the slope of the curve of $\log ms$ against $\log m/R$ can be seen in comparing the values for $\alpha = 0{\cdot}00$ and $\alpha = 0{\cdot}10$. While the former curve is extremely sensitive to changes in m_D, the latter is relatively insensitive, and even the theoretical estimate of m_D from leaf structure, obviously quite useless under hypostomatous conditions, has a qualitative utility under amphistomatous conditions that will increase as α increases.

CONCLUSION

The inherent variability of biological materials will not permit the verdict on the success of a mathematical analysis of their behaviour to be based on the comparison of only a few experimental results with theoretical predictions, so that the final decision as to the utility of the preceding theory must await the analysis of all the available experimental data. Although the comparison that has been made is satisfactory, the agreement between observed and estimated assimilation rates should be regarded as secondary to the main general argument. This has aimed at establishing two points:

(i) With the aid of some tedious arithmetic, curves such as those of figure 2 can be plotted; by the use of these curves the conductance of the stomata can be separated from the conductance of the leaf.

(ii) The process of separation involves the transitions $R \rightarrow m/R \rightarrow ms \rightarrow s$, i.e. is very much dependent upon the mesophyll resistance, m. The determination of m is the most important measurement involved, particularly in experiments using hypostomatous leaves with their stomata nearly fully open.

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Molecular structure in relation to oestrogenic activity: polynuclear analogues of $\gamma:\delta$-di-(4-hydroxyphenyl)-*n*-hexane ('hexoestrol')

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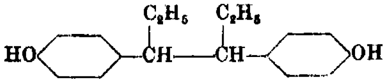
Eight of the most interesting polynuclear groups are shown to affect profoundly the activity of 'hexoestrol' on substitution for the 4-hydroxyphenyl groups. This evidence, combined with certain previously published results, demonstrates that 'hexoestrol' is almost certainly the most active member of the most potent series of oestrogens yet investigated. Further investigation is foreshadowed.

Introduction

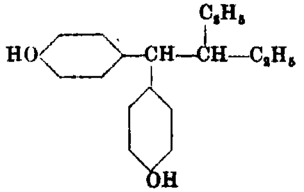
$\gamma:\delta$-Di-(4-hydroxyphenyl)-*n*-hexane (I) ('hexoestrol') is one of the most potent of all known oestrogens. It is therefore of importance to investigate the effects of modifications of the 'hexoestrol' molecule.

Dodds, Golberg, Lawson & Robinson (1939) have investigated alteration in length of the aliphatic part of the molecule in the cases of the closely related 4:4'-dihydroxy-$\alpha:\beta$-diethylstilbene and $\gamma:\delta$-di-(4-hydroxyphenyl) hexadiene, and have shown that the six-membered straight carbon chain with (4-hydroxy)-phenyl groups attached to the γ and δ carbons produces maximum potency.

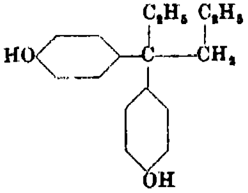
Two important isomers of 'hexoestrol' (I) have been described in an earlier publication (Campbell 1940). These are $\alpha:\alpha$-di-(4-hydroxyphenyl)-β-ethyl-butane (II) and $\gamma:\gamma$-di-(4-hydroxyphenyl)-*n*-hexane (III). (II) is fifty thousand times, and (III) five thousand times less active than 'hexoestrol'.



(I)



(II)



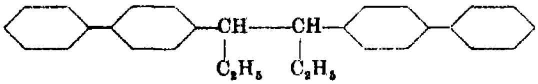
(III)

Of the remaining variations two types appear to merit greatest consideration. The first involves nuclear substitution on the benzene ring, a variation which produced extremely interesting results in the series of substituted di-(4-hydroxyphenyl)-methane derivatives studied in the publication mentioned above (Campbell 1940). A preliminary indication of the effect of this variation is given later.

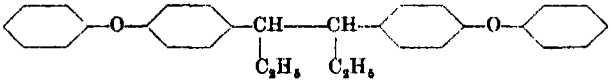
The second concerns substitution of other aromatic groups for the benzene rings in 'hexoestrol'.

INVESTIGATION OF NEW SERIES

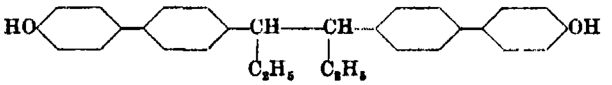
Of the groups chosen four were xenyl (IV), 4′-hydroxyxenyl (VI), 4-phenoxyphenyl (V) and 4-(4-hydroxyphenoxy)-phenyl (VII):



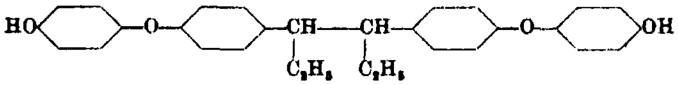
(IV)



(V)



(VI)

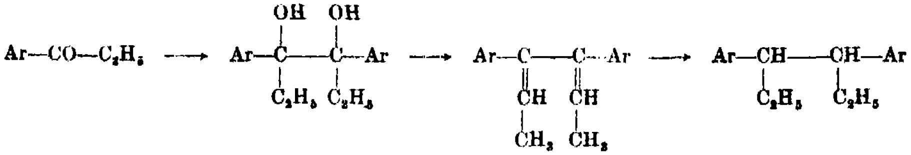


(VII)

With a limited, though very important, number of exceptions, the 4-hydroxyphenyl group is characteristic of substances showing oestrogenic activity. Many of the exceptions are hydrocarbons such as have been shown by Stroud (1940) to become oxidized in the animal body to paraphenols. Simple compounds containing the 4-hydroxyphenyl group are inactive, e.g. *p*-cresol, quinol and phenol itself, but activity is shown by 4:4′-dihydroxydiphenyl and by 4:4′-dihydroxydiphenylether (Dodds & Lawson 1938). 4-Hydroxydiphenyl is inactive in normal doses but 4-hydroxydiphenylether has activity approximately equal to that of the dihydroxy compound.

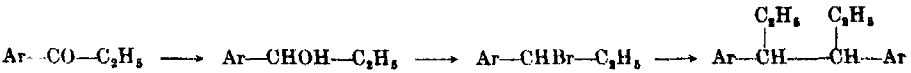
The first general method attempted was one used by Campbell, Dodds &

Lawson (1940) to prepare 'hexoestrol', methyl groups protecting phenolic hydroxyls when required:



$$\mathrm{Ar{-}CO{-}C_2H_5} \longrightarrow \mathrm{Ar{-}\underset{\substack{| \\ C_2H_5}}{\overset{\substack{OH \\ |}}{C}}{-}\underset{\substack{| \\ C_2H_5}}{\overset{\substack{OH \\ |}}{C}}{-}Ar} \longrightarrow \mathrm{Ar{-}\underset{\substack{\| \\ CH \\ | \\ CH_3}}{C}{-}\underset{\substack{\| \\ CH \\ | \\ CH_3}}{C}{-}Ar} \longrightarrow \mathrm{Ar{-}\underset{\substack{| \\ C_2H_5}}{CH}{-}\underset{\substack{| \\ C_2H_5}}{CH}{-}Ar}$$

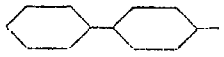
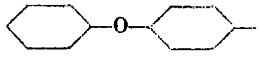
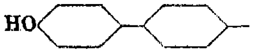
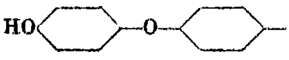
The four compounds desired, (IV), (V), (VI) and (VII), were all attempted by this method, but, without exception, the process broke down at the last step. The four dienes proved impossible to hydrogenate without pressure, and stubbornly resisted all endeavours to reduce under the most drastic conditions, although in one case (VII) half-hydrogenation took place, yielding a product with one ethylenic double bond. The method was then abandoned in favour of one based on that used by Bernstein & Wallis (1940) in the preparation of 'hexoestrol'. A Wurtz reaction has been carried out on anethole hydrobromide prepared from anethole, but the bromides were found to be readily obtainable direct from the carbinols:



$$\mathrm{Ar{-}CO{-}C_2H_5} \longrightarrow \mathrm{Ar{-}CHOH{-}C_2H_5} \longrightarrow \mathrm{Ar{-}CHBr{-}C_2H_5} \longrightarrow \mathrm{Ar{-}\overset{\substack{C_2H_5 \\ |}}{CH}{-}\overset{\substack{C_2H_5 \\ |}}{CH}{-}Ar}$$

This met with general success, although yields varied considerably. Phenolic hydroxyl groups were protected by the use of methyl ethers, and the final products were demethylated by heating in a sealed tube with potassium hydroxide and alcohol. Full details are given in the experimental section, together with an account of all new substances isolated in the unsuccessful application of method I. Biological tests were carried out according to the method of Allan, Dickens & Dodds (1930), and the oestrogenic activities are given in terms of an arbitrary rat unit, defined by Campbell (1940) as the minimum total weight of substance required to give 100 % full oestrus response in ovariectomized female rats when injected in six doses in sesame oil solution during 3 days.

Series 1

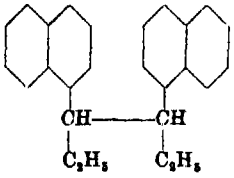
	group replacing 4-hydroxyphenyl groups in 'hexoestrol'	oestrogenic activity of product (r.u./g.)
IV		nil
V		nil
VI		nil
VII		70

It will be seen that in this series activity is only apparent when Ar = 4-(4-hydroxyphenoxy)-phenyl. This is the only group of the four which itself shows oestrogenic activity when completed by addition of one hydrogen atom, in this case giving 4-hydroxydiphenylether.

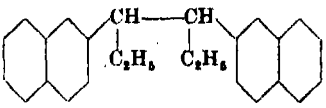
It is inferred that for high oestrogenic activity these groups cannot replace the 4-hydroxyphenyl group in the 'hexoestrol' molecule, notwithstanding their ability to produce simple active substances by addition of hydroxy groups only. It is also noteworthy that while inactive phenol is converted into a feebly active substance by preparation of its 4-hydroxyphenyl ether, similar treatment of hexoestrol lowers its potency five hundred thousand times.

The α-naphthyl group has long been of interest, since in the series of 1:2-dialkylacenaphthenediols and 2:2-dialkylacenaphthenones activity has only yet been found when the alkyl (or aryl) group is α-naphthyl. This series was first studied by Dodds & Lawson (1938) who prepared the di-*n*-propyl, diphenyl, dibenzyl and di-α-naphthylacenaphthenediols and the diphenyl and di-α-naphthylacenaphthenones. The series is now extended by the preparation and testing of the dimethyl, diethyl and di-(*p*-methoxyphenyl)-acenaphthenediols and the di-(*p*-methoxyphenyl) and di-(*p*-hydroxyphenyl)-acenaphthenones, but only the α-naphthyl derivatives show any activity. A parallel case exists in the activity of diphenyl-α-naphthyl carbinol, and the inactivity of diphenyl-β-naphthyl carbinol, of triphenyl carbinol and of 4-hydroxytriphenyl carbinol (Dodds & Lawson 1938).

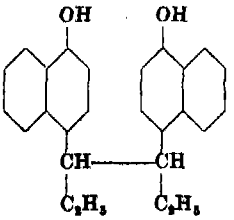
Hence the α- and β-naphthyl analogues (VIII and IX) of 'hexoestrol' have now been prepared, together with the 4-hydroxy-1-naphthyl analogue (X), which bears a close resemblance to 'hexoestrol', and the 6-hydroxy-2-naphthyl analogue (XI):



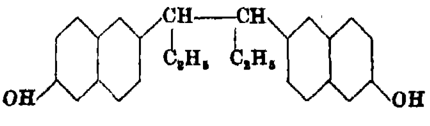
VIII



IX



X

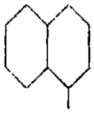
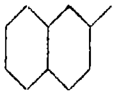
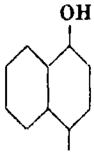
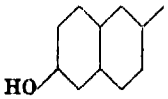


XI

It will be observed that the aromatic groups of (XI) are orientated in the same manner as rings *A* and *B* of the natural oestrogens oestrone, oestradiol, etc.

(XI) was first attempted by method I, but it was found impossible to obtain a pure product by dehydration of the pinacol from (6-methoxy-2-naphthyl)-ethyl-ketone and not possible to prepare any pinacol from (6-hydroxy-2-naphthyl)-ethyl-ketone. (VIII), (IX), (X) and (XI) were eventually all prepared by method 2, the *β*-naphthyl derivatives being obtained in better yield that the *α*-naphthyl. *α*- and *β*-methoxynaphthalene were used as starting materials for the preparation of (X) and (XI), and the technique adopted for the Friedel-Crafts reaction on *β*-methoxynaphthalene was found preferable to that of Haworth & Sheldrick (1934). (X) was difficult to obtain, and the final state of purity both of the phenol and its methyl ether was by no means high. It was, however, found inactive—a result upon which small quantities of impurity could have no bearing.

Series 2

	group replacing 4-hydroxyphenyl groups in 'hexoestrol'	oestrogenic activity of product (r.u./g.)
VIII		500
IX		25
X		nil (less than 10)
XI		8000

Once again, none of these groups produces an activity of the same order as the 4-hydroxyphenyl group in 'hexoestrol'. The highest activity produced occurs in (XI): this is very interesting in view of the connexion between the aromatic nucleus and the *A* and *B* rings of oestradiol, also the symmetry of the 6-hydroxy-2-naphthyl group which is comparable to that of the 4-hydroxyphenyl group.

The relation between di-α-naphthyl and di-β-naphthyl hexanes follows the example of the naphthyl-di-phenyl carbinols. The inactivity of (X) was rather surprising, since it may be regarded as 2:3:2′:3′-dibenz 'hexoestrol'.

Investigation of the relationship existing between the 4-hydroxyphenyl group and naphthyl and hydroxynaphthyl groups is proceeding.

CONCLUSIONS

In addition to the several points of interest raised by the study of the oestrogenic activity of the eight 'hexoestrol' analogues and already commented upon, there is the general conclusion that the 4-hydroxyphenyl group seems essential for maximum activity in this type of compound. Since past work has indicated the necessity for one particular arrangement of the groups present in 'hexoestrol', and it seems most unlikely that alteration of chain length will do otherwise than reduce activity, one may say that only one obvious point requires investigation before 'hexoestrol' is established as the most active of a very large class of oestrogens. This point concerns the effect of nuclear substitution on the benzene ring, with special reference to methyl groups in the 3 and 3′ positions. The only evidence yet available—that of the activity of γ:δ-di-(4-hydroxy-3-methylphenyl)-γ:δ-hexandiol compared with that of γ:δ-di-(4-hydroxyphenyl)-γ:δ-hexandiol—suggests that addition of methyl groups in these positions in the 'hexoestrol' molecule will lower the oestrogenic activity.

γ:δ-Di-(4-hydroxyphenyl)-*n*-hexane exists in two forms (Campbell *et al.* 1940), the high-melting isomer, 'hexoestrol', having an oestrogenic activity very much higher than that of the lower melting isomer. It is considered probable that all of the analogues described above have the same configuration as 'hexoestrol', since the higher melting isomer appears to be the only one obtained in the preparation of 'hexoestrol' by the Wurtz reaction. It has been shown by Carlisle & Crowfoot (1941) that 'hexoestrol' conforms to the meso configuration.

EXPERIMENTAL

4-*Phenylpropiophenone* (see also Willgerodt & Scholtz 1910). Propionic anhydride (45 g.) was added gradually to a mixture of diphenyl (50 g.) and aluminium chloride (100 g.) in nitrobenzene (250 c.c.), cooled in ice with mechanical stirring, and allowed to stand at room temperature overnight. The mixture was poured into dilute hydrochloric acid and ice, and worked up in the usual manner, m.p. 98°. Yield 35 g.

γ:δ-*Dixenyl*-γ:δ-*hexandiol.* 4-Phenylpropiophenone (35 g.) was reduced in moist ether by the amalgam from 85 g. aluminium foil (Vogel 1927).

The ether solution was filtered from aluminium hydroxide, dried, and the solvent removed. The residue was crystallized from xylene, m.p. 240°. (Found: C, 85·4; H, 7·15. $C_{30}H_{30}O_2$ requires C, 85·25; H, 7·15 %.) Yield 6 g.

γ:δ-Dixenyl-$\Delta^{\beta\delta}$-hexadiene. The above pinacol (100 mg.) was dropped on to molten potassium bisulphate (300 mg.) and heated at 200° for 10 min. The mixture was cooled, ground up, and extracted with ether. The ether solution was evaporated, the residue crystallized from light petroleum (60/80°), and crystallized from *cyclo*hexane, and from acetic acid, m.p. 151°. (Found: C, 93·2; H, 6·7. $C_{30}H_{26}$ requires C, 93·2; H, 6·8 %.) Yield 30 mg.

[α-Xenyl-α-propanol.] 4-Phenylpropiophenone (60 g.) was heated with aluminium *iso*propoxide (35 g.) and dry *iso*propyl alcohol under a fractionating column, the acetone formed being permitted to distil off slowly. When no acetone could be detected in the distillate (tested with 2:4-dinitrophenylhydrazine), the mixture was cooled, poured into dilute sulphuric acid and water, and extracted with ether. The ether solution was washed, dried and evaporated. The solid residue was used crude for the next stage. It consisted of a mixture of the required carbinol and its dehydration product.

γ:δ-Dixenylhexane. The above crude carbinol (60 g.) was dissolved in toluene (125 c.c.), the solution cooled in a freezing mixture below −5° and saturated with hydrogen bromide. The solution was decanted, dried over calcium chloride and run slowly into a stirred suspension of powdered sodium (25 g.) in dry toluene (200 c.c.) maintained at 55–60°. Stirring at this temperature was continued for 8 hr. and the whole allowed to remain at room temperature for 40 hr. Alcohol was added and the solvents removed in a current of steam. The residue was extracted with ether and the ether-insoluble portion recrystallized from benzene, m.p. 254°. (Found: C, 92·45; H, 7·7; M.W. 363. $C_{30}H_{30}$ requires C, 92·25; H, 7·75 %; M.W. 390.) Yield 5 g.

4-*Phenoxypropiophenone.* Propionic anhydride (16 g.) was added slowly to a mixture of diphenylether (20 g.) and aluminium chloride (35 g.) in nitrobenzene (50 c.c.), cooled in ice and mechanically stirred. The mixture was allowed to stand overnight and worked up in the usual manner. The crude product was crystallized from light petroleum (60/80°), m.p. 38°. (Found: C, 79·5; H, 6·4. $C_{15}H_{14}O_2$ requires C, 79·65; H, 6·25 %.) Yield 14 g.

γ:δ-Di-(4-phenoxyphenyl)-γ:δ-hexandiol. 4-Phenoxypropiophenone (2·2 g.) was reduced in moist ether by the amalgam from 5 g. aluminium foil. The ether solution was filtered from aluminium hydroxide, dried and evaporated. The gummy residue crystallized slowly from benzene-light petroleum (60/80°) and was recrystallized from benzene-light petroleum (1:2 by vol.) and from cyclohexane, m.p. 175°. (Found: C, 79·75; H, 6·5. $C_{30}H_{30}O_4$ requires C, 79·4; H, 6·6 %.) Yield 0·5 g.

γ:δ-Di-(4-phenoxyphenyl)-$\Delta^{\beta\delta}$-hexadiene. The above pinacol (0·5 g.) was heated under reflux with acetic anhydride (10 c.c.) and acetylchloride (5 c.c.) for 17 hr. The mixture was poured into water, extracted with ether and the etherial solution washed with water, sodium bicarbonate solution and finally water. On removal of ether the product crystallized, and was recrystallized from alcohol, m.p. 132–133°. (Found: C, 86·25; H, 6·8. $C_{30}H_{26}O_2$ requires C, 86·1; H, 6·3 %.) Yield 0·1 g.

γ:δ-Di-(4-phenoxyphenyl)-Δ^{x}-hexene. The above diene (0·5 g.) was dissolved in acetic acid and shaken in an atmosphere of hydrogen in the presence of platinum (0·2 g.) on charcoal. 40 c.c. hydrogen were absorbed. The solution was filtered, solvent removed and the residue crystallized from alcohol, m.p. 118°. (Found: C, 85·2; H, 6·7. $C_{30}H_{28}O_2$ requires C, 85·7; H, 6·7 %.) Yield 0·24 g.

α-(4-Phenoxyphenyl)-α-propanol. 4-Phenoxypropiophenone (113 g.) was reduced by heating under a fractionating column with aluminium *iso*propoxide (62 g.) and dry *iso*propylalcohol (320 c.c.) as before. The crude liquid carbinol was employed for the next stage, but a small portion was distilled at low pressure, b.p. 150°/0·2 mm. (Found: C, 79·3; H, 7·15. $C_{15}H_{16}O_2$ requires C, 78·9; H, 7·05 %.) Yield 115 g. crude.

γ:δ-Di-(4-phenoxyphenyl)-hexane. The above crude carbinol (45 g.) was dissolved in toluene (100 c.c.), converted to the bromide and submitted to the Wurtz reaction as before (22·5 g. sodium—10 hr. at 55–60°). Alcohol was added and the solvents removed by steam distillation. The solid residue was extracted with ether and the insoluble residue recrystallized from light petroleum (60/80°), m.p. 142°. (Found: C, 84·8; H, 6·95; M.W. 379. $C_{30}H_{30}O_2$ requires C, 85·3; H, 7·15 %; M.W. 422.) Yield 4 g.

4-(4-Methoxyphenyl)-propiophenone. Propionic anhydride (4·1 g.) was added slowly to a mixture of 4-methoxydiphenyl (5·3 g.) and aluminium chloride (9 g.) in tetrachloroethane (45 c.c.). The mixture was left overnight and poured into dilute hydrochloric acid and ice. The product was worked up in the usual manner and was crystallized from alcohol and from *cyclo*hexane, m.p. 143°. (Found: C, 79·9; H, 6·7. $C_{16}H_{16}O_2$ requires C, 80·0; H, 6·6 %.) Yield 3·2 g.

γ:δ-Di-[4-(4-methoxyphenyl)-phenyl]-γ:δ-hexandiol (two forms). The above ketone (3 g.) was reduced in moist ether by the amalgam from 6·25 g. aluminium foil. The ether solution was filtered, dried and evaporated. The residue crystallized slowly in contact with light petroleum (60/80°) and was recrystallized from *cyclo*hexane, m.p. 178°. (Found: C, 79·7; H, 7·1. $C_{32}H_{34}O_4$ requires C, 79·6; H, 7·1 %.) Yield 0·2 g. The ether-insoluble residue was treated with dilute hydrochloric acid, the solution filtered and the dried residue recrystallized from xylene and from anisole, m.p. 264°. (Found: C, 79·7; H, 7·1. $C_{32}H_{34}O_4$ requires C, 79·6; H, 7·1 %.) Yield 0·4 g.

γ:δ-Di-[4-(4-*methoxyphenyl*)-*phenyl*]-*$\Delta^{\beta\delta}$-hexadiene*. The pinacol (either form) (0·3 g.) was dropped on to molten potassium bisulphate (0·9 g.) and heated at 200° for 15 min. The melt was cooled, ground up and extracted with ether. The ether solution was evaporated, the product crystallized from acetic acid and recrystallized from methyl acetate, m.p. 152°. (Found: C, 85·5; H, 7·0. $C_{32}H_{30}O_2$ requires C, 86·0; H, 6·8 %.) Yield 0·2 g.

α-4-(4-*Methoxyphenyl*)-*phenyl-α-propanol*. 4-(4-Methoxyphenyl)-propiophenone (30 g.) was reduced by heating under a fractionating column with aluminium *iso*propoxide (15 g.) and *iso*propylalcohol (90 c.c.) as before. The crystalline product was recrystallized from light petroleum (60/80°), m.p. 93°. (Found: C, 79·4; H, 7·5. $C_{18}H_{18}O_2$ requires C, 79·3; H, 7·5 %.)

γ:δ-Di-4-(4-*methoxyphenyl*)-*phenyl-hexane*. The above carbinol (6·5 g.) was dissolved in toluene (50 c.c.), converted to the bromide and subjected to the Wurtz reaction as before (6·5 g. sodium—7 hr. at 50–60°). Alcohol was added and the solvents removed in a current of steam. The gummy residue crystallized in contact with light petroleum (60/80°) and the product was recrystallized from benzene, m.p. 260°. (Found: C, 85·1; H, 7·3. $C_{32}H_{34}O_2$ requires C, 85·3; H, 7·6 %.) Yield 0·1 g.

4-*Methoxy*-4′-*propenyl diphenyl*. The light petroleum mother liquors from the above were concentrated, and deposited crystals which were recrystallized from light petroleum four times, m.p. 150°. (Found: C, 85·3; H, 7·2. $C_{16}H_{16}O$ requires C, 85·7; H, 7·2 %.) The substance showed unsaturated properties.

γ:δ-Di-[4-(4-*hydroxyphenyl*)-*phenyl*]-*hexane*. The methyl ether (0·2 g.) was heated in a sealed tube with potassium hydroxide (1 g.) and absolute alcohol (2 c.c.) for 18 hr. at 210°. The tube was cooled, its contents diluted with water and extracted with ether. The aqueous solution was acidified and the precipitated phenol collected and recrystallized from benzene, m.p. 240°. (Found: C, 85·15; H, 6·95. $C_{30}H_{30}O_2$ requires C, 85·25; H, 7·15 %.) Yield 0·07 g.

4-(4-*Methoxyphenoxy*)-*propiophenone*. Propionic anhydride (6 g.) was added slowly to 4-methoxydiphenylether (9 g.) and aluminium chloride (13·4 g.) in 50 c.c. tetrachloroethane, cooled in ice and mechanically stirred. The mixture was left overnight at room temperature, then poured into ice and hydrochloric acid. The solvent was removed in a current of steam, and on cooling, the residue solidified. The product was collected and recrystallized from methyl alcohol and from light petroleum (60/80°), m.p. 64°. (Found: C, 75·2; H, 6·5. $C_{16}H_{16}O_3$ requires C, 75·0; H, 6·3 %.) Yield (once recrystallized) 7 g.

γ:δ-Di-[4-(4-*methoxyphenoxy*)-*phenyl*]-*γ:δ-hexandiol*. The above ketone (7 g.) was reduced in moist ether by the amalgam from 13·7 g. aluminium foil. The ether solution was filtered and evaporated. The residue crystallized in contact with light petroleum and was recrystallized from benzene, m.p. 200°. A further crop was obtained by treatment of the ether-insoluble portion with dilute hydrochloric acid. (Found: C, 75·2; H, 6·7. $C_{32}H_{34}O_6$ requires C, 74·7; H, 6·6 %.) Yield 1·2 g.

[*α-4-(4-Methoxyphenoxy)-phenyl-α-propanol.*] 4-(4-Methoxyphenoxy)-propiophenone (20 g.) was reduced with aluminium *iso*propoxide (10 g.) and *iso*propylalcohol (60 c.c.) as before. The product was distilled at reduced pressure, b.p. 182–188°/1 mm. Yield 18 g. (Attempted redistillation resulted in dehydration and polymerization.) The product contained some dehydration product and was used for the next stage without further purification.

γ:δ-Di-[4-(4-methoxyphenoxy)-phenyl]-hexane. α-4-(4-Methoxyphenoxy)-phenyl-α-propanol (20 g.) was dissolved in toluene (75 c.c.), converted to the bromide and subjected to the Wurtz reaction as before (4·5 g. sodium —6 hr. at 55–60°). Alcohol was added and the solvents then removed in a current of steam. The residue deposited crystals from light petroleum and the product was recrystallized from the same solvent, m.p. 146°. (Found: C, 79·7; H, 6·9. $C_{32}H_{34}O_4$ requires C, 79·6; H, 7·1 %.) Yield 1·2 g.

γ:δ-Di-[4-(4-hydroxyphenoxy)-phenyl]-hexane. The methyl ether (0·3 g.) was heated in a sealed tube with potassium hydroxide (1 g.) and absolute alcohol (2 c.c.) for 18 hr. at 200°. The product was worked up as before. The phenolic product was recrystallized from benzene, m.p. 198°. (Found: C, 79·5; H, 6·9. $C_{30}H_{30}O_4$ requires C, 79·3; H, 6·7 %.) Yield 0·06 g.

6-Methoxy-2-propionylnaphthalene (see also Haworth & Sheldrick 1934). A mixture of aluminium chloride (17 g.) and nitrobenzene (44 c.c.) was cooled in ice and to this was added slowly, with stirring, a mixture of β-methoxynaphthalene (10 g.) and propionyl chloride (5·85 g.) in nitrobenzene (40 c.c.). Stirring was continued overnight at room temperature and the mixture poured into dilute hydrochloric acid and ice. Nitrobenzene was removed by steam distillation and the solid residue collected, dried and distilled at low pressure, b.p. 156–160°/0·4 mm. The product was recrystallized from light petroleum (60/80°), m.p. 109°. Yield 4·1 g.

γ:δ-Di-(6-methoxy-2-naphthyl)-γ:δ-hexandiol. 6-Methoxy-2-propionylnaphthalene (3·8 g.) was reduced in moist ether with the amalgam from 9·1 g. aluminium foil. The ether solution was filtered and evaporated. The resinous residue crystallized in contact with benzene-light petroleum and was recrystallized from benzene and from toluene, m.p. 248°. (Found: C, 78·1; H, 7·3. $C_{28}H_{30}O_4$ requires C, 78·1; H, 7·0 %.) Yield 0·5 g.

6-Hydroxy-2-propionylnaphthalene. Aluminium bromide (20 g.) in dry benzene (150 c.c.) was added to a solution of 6-methoxy-2-propionylnaphthalene (5 g.) in dry benzene (100 c.c.), the mixture allowed to stand for 15 min., and heated under reflux for 3½ hr. The mixture was cooled and poured into dilute hydrochloric acid and ice. The benzene layer was separated, the aqueous extracted with ether, and the washed benzene and ether solutions mixed and extracted with sodium hydroxide solution (2N). The alkaline solution was acidified, the precipitated phenol extracted with ether, and the ether solution washed, dried and evaporated. The product was recrystallized from toluene, m.p. 158°. (Found: C, 78·15; H, 5·75. $C_{13}H_{12}O_2$ requires C, 77·95; H, 6·0 %.) Yield 2 g.

α-(6-Methoxy-2-naphthyl)-α-propanol. 6-Methoxy-2-propionylnaphthalene (24 g.) was reduced with aluminium *iso*propoxide (14·6 g.) and *iso*propyl alcohol (60 c.c.). The product solidified (yield 26·3 g. crude) and was recrystallized from low-boiling light petroleum (40/60°), m.p. 50°. (Found: C, 77·6; H, 7·8. $C_{14}H_{16}O_2$ requires C, 77·7; H, 7·5 %.)

γ:δ-Di-(6-methoxy-2-naphthyl)-hexane. α-(6-Methoxy-2-naphthyl)-α-propanol (8·5 g.) was dissolved in toluene (40 c.c.), converted to the bromide and subjected to the Wurtz reaction as before (4 g. sodium—7 hr. at 50–60°). Alcohol was added and the solvents removed by steam distillation. The residue crystallized in contact with light petroleum (60/80°) and was recrystallized from benzene-light petroleum (3:1 by vol.), m.p. 255°. (Found: C, 84·3; H, 7·2. $C_{28}H_{30}O_2$ requires C, 84·4; H, 7·6 %.) Yield 0·5 g.

6-Methoxy-2-propenylnaphthalene. The above Wurtz reaction was attempted at higher temperature (60–70°). No Wurtz product was obtained, but loss of hydrogen bromide occurred, yielding a solid which was recrystallized from light petroleum, m.p. 96°. (Found: C, 84·6; H, 7·35. $C_{14}H_{14}O$ requires C, 84·8; H, 7·1 %.) The product had unsaturated properties.

γ:δ-Di-(6-hydroxy-2-naphthyl)-hexane. The methyl ether (0·7 g.) was heated in a sealed tube with potassium hydroxide (3 g.) and absolute alcohol (6 c.c.) for 16 hr. at 210°. The tube was cooled and the contents worked up as usual. The product was recrystallized from benzene, m.p. 253°. (Found: C, 84·35; H, 6·85. $C_{26}H_{26}O_2$ requires C, 84·3; H, 7·1 %.) Yield 0·18 g.

4-Methoxy-1-propionylnaphthalene (see also Gattermann, Ehrhardt & Maisch 1890). α-Methoxynaphthalene (125 g.), propionic anhydride (125 g.) and carbon disulphide (250 c.c.) were mixed and cooled in ice, with stirring. Aluminium chloride (250 g.) was slowly added (2 hr.), the mixture stirred overnight and worked up in the usual manner. Yield 92 g.

α-(4-Methoxy-1-naphthyl)-α-propanol. 4-Methoxy-1-propionylnaphthalene (100 g.) was reduced with aluminium *iso*propoxide (60 g.) and *iso*propyl alcohol (300 c.c.) as before. The crude liquid product was employed for the next stage, but a small portion was distilled at low pressure, b.p. 150°/0·2 mm., and then crystallized. The product was recrystallized from light petroleum (60/80°), m.p. 68–70°. (Found: C, 78·1; H, 7·45. $C_{14}H_{16}O_2$ requires C, 77·75; H, 7·45 %.)

γ:δ-Di-(4-methoxy-1-naphthyl)-hexane. The above carbinol (50 g.) was dissolved in toluene (150 c.c.), converted to the bromide and subjected to the Wurtz reaction (25 g. sodium—12 hr. at 55–60°). The product was worked up as before and crystallized in contact with light petroleum (60/80°). The crystallized product was recrystallized from benzene, m.p. 209°. (Found: C, 84·8; H, 6·9; M.W. 366. $C_{28}H_{30}O_2$ requires C, 84·4; H, 7·6 %; M.W. 398.) Yield 2·6 g. (not satisfactorily purified).

γ:δ-Di-(4-hydroxy-1-naphthyl)-hexane. The methyl ether (1·5 g.) was heated in a sealed tube with potassium hydroxide (4 g.) and absolute alcohol (8 c.c.) for 17 hr. at 200°. The product was worked up as usual and recrystallized from benzene and from toluene, m.p. 224°. (Found: C, 84·65; H, 6·5. $C_{26}H_{26}O_2$ requires C, 84·3; H, 7·05 %.) Yield 0·2 g.

1-*Propionylnaphthalene* was prepared according to Rousset (1897), and, although purified through the picrate, was not found with any admixture of 2-isomer. Yield 22 %.

[*α-*(1-*Naphthyl*)-*α-propanol.*] 1-Propionylnaphthalene (80 g.) was reduced with aluminium *iso*propoxide (50 g.) and *iso*propyl alcohol (250 c.c.) as before. The crude liquid carbinol was employed for the next stage. Yield 84 g. crude.

γ:δ-Di-(1-*naphthyl*)-*hexane.* The above crude carbinol (40 g.) was dissolved in toluene (120 c.c.), converted to the bromide and subjected to the Wurtz reaction as before (20 g. sodium—20 hr. at 50–60°). The product was distilled at low pressure and a fraction collected, b.p. between 100 and 160°. It was crystallized from low-boiling light petroleum (40/60°) and was recrystallized from light petroleum (60/80°), m.p. 155°. (Found: C, 92·2; H, 7·75; M.W. 357. $C_{26}H_{26}$ requires C, 92·25; H, 7·75 %; M.W. 338.) Yield 0·5 g.

2-*Propionylnaphthalene* (see under 1-propionylnaphthalene). A mixture of powdered naphthalene (130 g.), aluminium chloride (300 g.) and nitrobenzene (1 l.) was stirred and cooled in ice. Propionic anhydride (130 g.) was slowly added, the mixture stirred for a further hour and left overnight at room temperature. The mixture was poured into dilute hydrochloric acid and ice, reactants removed by exhaustive steam distillation and the solid product recrystallized from light petroleum (60/80°). Yield 42 %.

α-(2-*Naphthyl*)-*α-propanol.* 2-Propionylnaphthalene (55 g.) was reduced with aluminium *iso*propoxide and *iso*propyl alcohol as before. The product crystallized from low-boiling light petroleum (40/60°) and was recrystallized from the same solvent, m.p. 37°. (Found: C, 83·9; H, 7·3. $C_{13}H_{14}O$ requires C, 83·8; H, 7·6 %.) Yield 50 g. crude.

γ:δ-Di-(2-*naphthyl*)-*hexane.* The above crude carbinol (42 g.) was dissolved in toluene (200 c.c.), converted to the bromide and subjected to the Wurtz reaction as before (28 g. sodium—50° for 18 hr.). Alcohol was added and the solvents then removed by steam distillation. The residue was extracted with ether, the ether solution evaporated and the residue crystallized from low-boiling light petroleum (40/60°). The crystals were added to the ether-insoluble portion and recrystallized from benzene, m.p. 198°. (Found: C, 92·2; H, 7·5; M.W. 306. $C_{26}H_{26}$ requires C, 92·25; H, 7·75 %; M.W. 338.) Yield 3 g.

1:2-*Di-(p-methoxyphenyl)*-1:2-*acenaphthenediol.* Acenaphthenequinone (18·2 g.) suspended in dry ether (50 c.c.) was added gradually to an ice-cold Grignard solution prepared from *p*-bromoanisole (107 g.) and magnesium (13·9 g.) in ether (150 c.c.). The mixture was stirred and heated under reflux for 4 hr. and allowed to stand overnight. The reaction mixture was poured on to ice and ammonium chloride, worked up in the usual manner and recrystallized from alcohol, m.p. 162°. (Found: C, 78·35; H, 5·65. $C_{26}H_{22}O_4$ requires C, 78·35; H, 5·5 %.) Yield 22 g.

2:2-*Di-(p-methoxyphenyl)-acenaphthenone.* The above diol (5 g.) was heated under reflux with acetic acid (75 c.c.) for 1 hr.; hydrochloric acid (10 c.c.) and water (200 c.c.) were then added, in succession, to the hot solution, which was cooled and filtered. The precipitated solid was recrystallized from alcohol, m.p. 151°. (Found: C, 81·9; H, 5·4. $C_{26}H_{20}O_3$ requires C, 82·0; H, 5·3 %.) Yield 3·5 g. (The same product was obtained by heating the diol (1 g.) with formic acid (40 c.c.) for 4 hr. under reflux. Yield 0·5 g.)

2:2-*Di-(p-hydroxyphenyl)-acenaphthenone.* The methyl ether (1 g.) was dissolved in dry benzene (20 c.c.) and treated gradually with a suspension of aluminium chloride (4 g.) in dry benzene (30 c.c.). The mixture was heated under reflux for 4 hr., cooled and poured into dilute hydrochloric acid and ice. Benzene was removed by steam distillation and the phenolic product isolated in the usual manner, crystallized from anisole and from acetic acid (50 % by vol.), m.p. 240–241°. (Found: C, 81·3; H, 4·7. $C_{24}H_{16}O_3$ requires C, 81·8; H, 4·6 %.) Yield 0·2 g.

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On the dehiscence of the antheridium and the part played by surface tension in the dispersal of spermatocytes in Bryophyta

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[Plate 14]

In a large number, probably the majority, of Bryophyta the antheridium on dehiscence under water discharges the spermatocytes in a mass. This mass is pushed out by a liquid which accumulates in the base of the antheridium. On reaching an air-water surface the spermatocytes spread apart rapidly and form a layer on the surface in which they are regularly spaced apart. This surface spread is evidently correlated with the presence of fat in the spermatocyte mass. This fat lowers the surface tension and causes the spreading. In *Sphagnum* and some liverworts where surface spreading does not occur fat has not been detected in the spermatocyte mass.

This spreading action is evidently of considerable biological significance and plays an important part in carrying a supply of spermatocytes rapidly to the neighbourhood of the female organ where the free sperms are liberated and are then directed chemotactically to the female gamete.

The distribution in a surface layer instead of throughout the liquid offers an explanation of why insects play an effective part in carrying spermatocytes from male plants to female plants in dioecious mosses.

1. Introduction

Since 1851, when Thuret described the antheridia of various Cryptogams and their mode of dehiscence, much has been written about the development, structure and mechanism of the Bryophyte antheridium. Relatively few observations have been made of the manner in which its contents are dispersed and fertilization effected. The phenomena, however, which have recently been observed by us indicate that the mechanism of discharge and dispersal of the contents are of a complex and varied character, and while we are able to put on record some interesting observations it is clear that there are several directions in which research might be pursued on the physiology of these processes.

Previous work has shown that there are considerable differences in the way in which the antheridium dehisces and the contents distributed. To take for example *Fimbriaria*; Pierce (1902) showed that the contents of the antheridium were explosively discharged into the air, and Cavers (1903) observed the same behaviour in *Conocephalum* (*Fegatella*). The mass of spermatocytes in these two plants is thrown to a height of several centimetres from the male plant. In contrast to this, in *Sphagnum*, the apex of the antheridium opens and the membranes of the spermatocytes thus exposed immediately rupture and liberate the free-swimming sperms. In *Sphagnum*, therefore, the transport of the sperm to the archegonium must be effected by the activity of the sperm itself. Gayet (1897, p. 229) showed that in dioecious mosses fertilization may be effected by the transport of male gametes on insects or by the splashing of rain. Harvey-Gibson & Miller-Brown (1927) have recorded the different types of insects and mites found in the perichaetial cups and archegonial branches of *Polytrichum*, and observed that there were spermatocytes entangled in the hairs of these creatures. If the transport of sperms depends on the accidental adherence of free-swimming sperms to the legs of insects crawling over the perichaetial cups it is difficult to understand how it is so effective. If a hair is dipped into water in which there are suspended particles and withdrawn, very few if any of the particles will be found sticking to the hair.

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* The observations recorded in this memoir were made during the summers from 1938 to 1941. The junior author (H.M.) assisted in the work during the summer of 1940 and was responsible for most of the staining tests.

Free-swimming sperms have a very limited range of action unless their movements are controlled by chemotaxy; indeed, Showalter (1926, p. 701) has shown that while the spermatozoids of *Aneura pinguis* retain their motility for as much as 6 hr. at a temperature of between 20 and 25° C, they may travel but a very short distance from their point of liberation. He observed that if a large number of sperms were placed at one end of a small pool of water 1 cm. long and 0·5 cm. wide they were, with the exception of one or two individuals, still crowded at the end of the pool an hour later.

Taken alone, these phenomena appear to give a very unsatisfying explanation of how the sperms manage to pass from an antheridium to an archegonium which may be situated at some distance from it, and how such abundant crops of sporogonia are produced on the female plants of dioecious mosses.

Some years ago the senior author observed that when the mass of spermatocytes protruded from a ripe antheridium of *Mnium hornum* L. and was kept completely submerged in water the mass retained its coherence for several hours; in fact, neither the separation of the spermatocytes nor the liberation of free-swimming sperms was observed under these conditions. If, however, the spermatocyte mass was allowed to touch the free surface of the water the part of the mass actually in contact with the air-water surface immediately broke up and the spermatocytes were spread in a rapidly expanding film on the water surface. This observation led to wider investigations with the collaboration of the junior author in 1940.

The results of our investigations show that the surface distribution of spermatocytes is of widespread occurrence in the Musci and Hepaticae and is probably one of the most important factors ensuring fertilization. The dehiscence of the antheridium and the dispersal of the spermatocytes in *Mnium hornum* L. will be described first, as this moss is of common occurrence and its relatively large antheridia and perichaetia afford excellent material for experiment.

2. DEHISCENCE OF THE ANTHERIDIUM AND THE DISPERSAL OF THE SPERMATOCYTES IN *MNIUM HORNUM* L.

Mnium hornum L. is dioecious and the male and female plants as a rule form distinct cushions in moist shaded localities on the soil or on fallen tree trunks. Antheridial shoots differ from female and sterile shoots in their reflexed perichaetial leaves which form a distinct rosette. In normal seasons in the Glasgow district some antheridia mature in May and there is a succession of antheridia reaching maturity until the beginning of July. The ripe antheridia discharge their contents in wet weather. If this is followed by a dry spell the perichaetial leaves present a glistening appearance. This is due to some of the material discharged from antheridia drying and forming a film on the upper surfaces of the perichaetial leaves. This glistening appearance may persist right through the winter, and by it the male plants may be distinguished from the female at a distance of several yards.

If a drop of water* is placed in an antheridial cup in which there are mature antheridia dehiscence takes place in about 4 min. If the cup is viewed from above by means of a binocular microscope, small whitish cylindrical threads are seen to emerge from the bottom of the cup and slowly lengthen until they reach the surface of the water. When they reach the surface they may become less distinct owing to the spread of some of their substance over the surface of the drop.

If mature antheridia are dissected out and mounted separately in water on a slide under a coverslip and observed with a microscope the process of dehiscence can be followed in detail. As has already been observed by Goebel (1898) the antheridial wall is composed of a single layer of tabular cells containing chloroplasts. The apical or cap cell is roughly cubical and contains widely spaced chloroplasts. The chloroplasts in the antheridial wall tend to turn to a brown or orange colour when the antheridium is senescent. In the mature antheridium the mass of spermatocytes does not always fill the space within the wall, and there is sometimes a space containing a colourless fluid between the proximal end of the spermatocyte mass and the base of the antheridium.

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* In all the experiments described in this paper tap water was used. This water supply comes from Loch Katrine in Perthshire.

When placed in water the inner surfaces of the outer walls of the cap cell swell, owing to their mucilaginous nature, and the contents of the cell are forced inwards. The inner wall then bursts inwards and the whole content of the cell, including the chloroplasts, is forced on to the top of the spermatocyte mass. This phenomenon has been recorded by Goebel (1898) and, as he suggested, is probably due to the pressure set up in the cell owing to the taking up of water by the inner, mucilaginous layers of the outer wall of the cap cell. These layers stain intensely with methylene blue. The cap cell now appears clear and highly refringent.

A short time after these changes have taken place the outer wall of the cap cell is ruptured and the contents of the antheridium stream steadily through the perforated cap cell. The spermatocyte mass protrudes as a sausage-shaped mass into the water. The diameter of the perforation is about a quarter of the diameter of the spermatocyte mass inside the antheridium. The free part of the spermatocyte mass has a slightly greater diameter than the mass inside the antheridium but has approximately the same form when completely extruded. The mass may be completely extruded in from 4 to 10 min. and is seen to consist of spherical spermatocytes held together by a mucilaginous matrix. Tests with methylene blue show that the matrix contains mucilage. Each spermatocyte contains a sperm which moves actively inside the spermatocyte membrane. The motion of the sperms gives the whole extruded mass a shimmering appearance.

As the mass of spermatocytes leaves the antheridium it is evident that the space between the proximal end of the mass and the base of the antheridium becomes filled with a colourless fluid. The nature of this fluid has not yet been discovered. It may be mucilage formed from the wall cells at the base of the antheridium. Whatever its nature it must be the pressure exerted by this fluid which pushes out the spermatocyte mass. The wall of the antheridium is only slightly elastic and extensible because when the antheridium has discharged the spermatocyte mass there is no obvious diminution in the diameter of the antheridium. The water which forms the bulk of this propellent fluid must enter through the living cells of the wall round the base of the antheridium. The steady rate at which the contents of the antheridium are expelled is therefore probably due to the rate at which water passes through these living cells. That the fluid in the antheridium is under pressure is shown by the fact that sometimes when the last of the spermatocyte mass passes through the cap cell there is evidence of a short gush of liquid after it, pushing the whole mass a short distance away from the end of the antheridium (figure 1, plate 14).

If the spermatocyte mass is kept completely submerged in water no further changes are observed even after a lapse of several hours, and no free-swimming sperms have been observed. It is evident that the sperms are not liberated from the spermatocyte membrane for possibly several hours after the dehiscence of the antheridium. If, however, the experiment is so arranged that the spermatocyte mass reaches the air-water surface, the mass at its point or points of contact with the surface immediately breaks up and spreads with great rapidity, forming a film over the free surface of the water (figure 6 and figures 1, 2, plate 14).

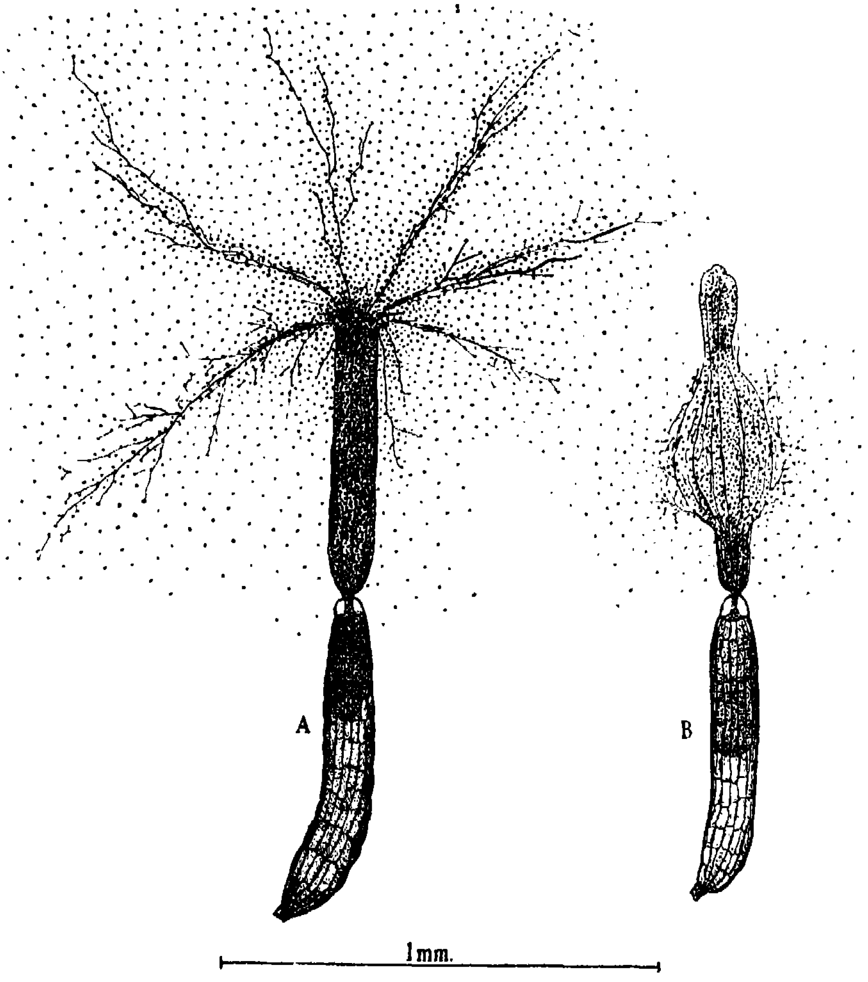


FIGURE 6. *Mnium hornum* L. Dehiscence of antheridium. A. Antheridium with about two-thirds of the contents extruded. Apex of extruded mass in contact with the air-water surface and strands of mucilage and spermatocytes spreading over the surface. B. Similar to A but with the middle of the extruded mass touching the surface.

If this expanding superficial film of spermatocytes is viewed from above (figure 3, plate 14) it can be seen that the spermatocytes behave as if they were repelling one another and at the same time to be connected together by colourless viscous strands of the mucilaginous matrix between them. Jerky movements of the spermatocytes and strands occur owing to the snapping of the strands connecting the spermatocytes. When the mass starts to spread (figure 6) it may be seen that the material forming the strands is aggregated in a number of main strands which break up as the spreading takes place. These main strands must be arranged parallel to one another in the spermatocyte mass inside the antheridium. It is possible, although it has not been confirmed, that these strands may correspond to the vertical rows of cells forming the wall of the antheridium. In the later stages of dispersion of the mass the individual spermatocytes are completely separated from one another and have no connecting strands. They are spaced with remarkable regularity over the surface.

It was observed that if only a very small drop of water was used the spermatocyte mass after some undetermined concentration of sperms had been spread on the surface stopped breaking up. It seemed that the surface had become saturated. This was proved by the addition of more water to increase the air-water surface. The rest of the spermatocyte mass then broke up and spread over the larger surface.

Lycopodium powder was dusted on to a water surface over a ripe antheridium. When the latter dehisced and its contents reached the surface the spores were swept in front of the film of spermatocytes and an area appeared clear from spores surrounding the point where the antheridium was placed.

TABLE 1. *MNIUM HORNUM* L.

reagent	fat reaction	reaction with antheridium	conclusion
Sudan III	pink	apical cells of antheridium faintly pink. Sperm cells definitely pink with pink globules in the mucilage between the spermatocytes	fat present in the sperm mass, especially in the mucilage, and traces in the apical cells of the antheridium
Sudan IV	red	antheridium apical cells faintly coloured. Spermatocyte mass definitely red	fat present in the sperm mass and traces of the fat in the apical cells of the antheridium
Nile blue sulphate	fat—pink	mucilaginous matrix pinkish purple	fat present in the mucilage
	fatty acid—blue	spermatocytes blue	fatty acid in the spermatocyte
osmic acid	black	spermatocyte mass black	fat present in the sperm mass

The behaviour of the spermatocyte mass on reaching a free water surface suggested that some substance with a low surface tension might be present in the mass and that the surface spread is comparable to the spread of an oil film on a water surface. Table 1 gives an account of the action of selected reagents on the antheridia and spermatocyte mass in an attempt to discover the nature of the agent causing the surface dispersal. The observations recorded in the table suggest that fat is present in small quantities in the spermatocyte mass.

3. NOTES ON THE DEHISCENCE OF THE ANTHERIDIUM AND THE DISTRIBUTION OF THE SPERMATOCYTES IN OTHER BRYOPHYTA

Sphagnales

Sphagnum cymbifolium Ehrh. Cavers (1910) described the dehiscence of the antheridium and fertilization of the archegonium. The antheridium opens by the swelling and rounding off of the cells at the apex which separate from one another. The cells at the sides curve outwards so that the open antheridium is campanulate in shape. The sperms are liberated immediately from the spermatocyte mass and swim about actively.

The tests applied gave no indication of the presence of fat or fatty acid either in the antheridium or sperms.

Bryales

(*a*) *Polytrichaceae.*

Catharinea undulata Web. & Mohr. The antheridia of *Catharinea* appear to mature slightly later in the season than the other mosses which have been studied by us. The dehiscence of the antheridium and the liberation and surface spread of the spermatocytes follows much the same lines as in *Mnium*, but the cells of the wall immediately adjacent to the apical cell also discharge their contents inwards before dehiscence occurs. Small highly refringent globules, possibly fat, were observed in the surface film after the discharge. If the dehiscence occurs in a solution of methylene blue the first material to be discharged, presumably the contents of the apical cells, and the apical cell itself are stained deeply.

Polytrichum commune L. As in *Mnium* the antheridia of *Polytrichum* are situated in the bottom of a cup formed by the perichaetial leaves. After rain this cup is usually found to contain water. The preliminary stages of dehiscence are like those observed in *Catharinea*; the cells which surround the apical cell as well as the apical cell itself have their contents forced inwards before dehiscence of the antheridium occurs. In the subsequent behaviour and the surface spread of spermatocytes this moss is similar to *Mnium*. Some antheridia, possibly those approaching senescence, discharge their spermatocytes in clumps at intervals. This behaviour may possibly be due to the existence of clefts filled with fluid which are sometimes visible, dividing the spermatocyte mass in the antheridium into separate parts.

On testing the antheridia and spermatocyte mass with Sudan III, Sudan IV and Nile blue sulphate there were clear indications of the presence of fat.

Harvey-Gibson & Miller-Brown (1927) give a list of insects and mites found in the antheridial cups of *Polytrichum*. These writers suggest that these creatures were searching for mucilage. Spermatocytes were found on their bodies.

Polytrichum juniperinum Willd. This moss exhibited the same phenomena as were observed in *P. commune*. Tests indicated that fat was present in the sperm mass.

(*b*) *Funariaceae.*

Funaria hygrometrica Sibth. The antheridia of *Funaria* are found in groups surrounded by a cup of perichaetial leaves at the apex of the shoot. Dehiscence takes place into water which collects in the cup. The cells forming the wall of the club-shaped antheridium contain large numbers of chloroplasts which turn bright reddish brown when the antheridium is ripe. In March, ripe antheridia were got from plants growing in a greenhouse. As in *Mnium* the contents of the cap cell slip through into the antheridial cavity before dehiscence occurs. The clear tip becomes swollen and soon bursts, and the spermatocyte mass flows steadily out.

As it reaches the surface, the spermatocyte mass spreads out in a film as described for *Mnium*.

On staining the spermatocyte mass with Sudan IV and Nile blue sulphate, a positive reaction for fat was obtained.

(c) *Bartramiaceae.*

Bartramia pomiformis Hedw. The apical cell and those immediately adjacent discharge their contents inwards before the antheridium dehisces. There is rapid discharge and the spermatocytes spread rapidly and evenly over the surface.

(*d*) *Bryaceae.*

Leptobryum pyriforme Wils. This small moss is synoecious. The apical cell and the cells around it discharge their contents inwards. The spermatocyte mass is discharged fairly rapidly and the surface spreading is rapid. Tests indicated the presence of fat in the sperm mass.

Bryum capillare L. When ripe antheridia were placed in a drop of water on a slide, dehiscence could be observed. The tip of the antheridium becomes clear as in *Mnium hornum* by the expulsion inwards of the contents of the cells at the apex. When the spermatocyte mass is extruded and reaches the water surface, the spermatocytes are spread over the surface.

When the water surface was dusted with Lycopodium powder the film of spermatocytes pushed the powder away from the surface round the antheridium. On testing with Nile blue sulphate positive indications of fat in the matrix and fatty acid in the spermatocytes were obtained.

On three occasions Orbatid mites were found in the antheridial cups.

Mnium hornum L. See p. 450.

Mnium punctatum L. The discharge of the spermatocyte mass from the antheridium and the surface spread of the spermatocytes was observed to occur in June and July, in the same manner as in *M. hornum*. The strands connecting the spermatocytes (figure 4, plate 14) responded to tests for mucilage and traces of fat.

(*e*) *Hookeriaceae*.

Pterigophyllum lucens Brid. Natural dehiscence was not observed but the spermatocyte mass was pressed out of an antheridium and surface spread of the spermatocytes was observed. This moss is autoecious. The male and female branches are very short and small and are almost completely concealed under the large overlapping complanate leaves.

Marchantiales

Conocephalum conicum (L.) Dum. Mature antheridial plants collected in June were placed under a bell jar in a greenhouse for a few days. They were kept moist by being laid on wet sand but no free water was applied to the upper surfaces of the plants. On a sunny morning the bell jar was removed and water was sprayed on to the surface of the plants. Within a few minutes spurts of what appeared to be white spray rose from the antheridiophores. Each spurt lasted for about half a second before it dispersed. The spurt rose vertically and at a height of about 8 cm. the disintegration into spray was complete.

Glass slides were held horizontally over the antheridiophores at heights of about 2·5 and 8 cm., and it was found that the discharged material adhered to them. The spermatocyte mass is evidently ejected with a high initial velocity and breaks up into a spray of small droplets (figure 7). Each droplet consists of liquid containing a number of spermatocytes; the smallest droplet observed adhering to the slides contained five. At a distance of 8 cm. above the antheridium the majority of the droplets contained about fifty spermatocytes. It is possible that the finer droplets had too low a velocity to cause them to adhere.

If water is added to the droplets adhering to the slides the sperms are liberated from the spermatocytes within a few minutes. There is no evidence of surface spreading by the spermatocytes and no appreciable amount of fat appears to be present among them.

Marchantia polymorpha L. The antheridia of *M. polymorpha* are confined in narrow cavities which open to the surface on the top of the disk-like head of the antheridiophore. When the antheridia are ripe the disk is slightly concave and Strasburger (1869, p. 419) observed that when a drop of water is allowed to fall on it the drop spreads over the surface. The discharge of the antheridia causes the drop to appear milky. He suggested that this water laden with sperms gets splashed by rain on to the female plants. The archegoniophores of the latter have a convex upper surface and water dropped on to them flows over the edge of the archegonia which are situated on the under surface.

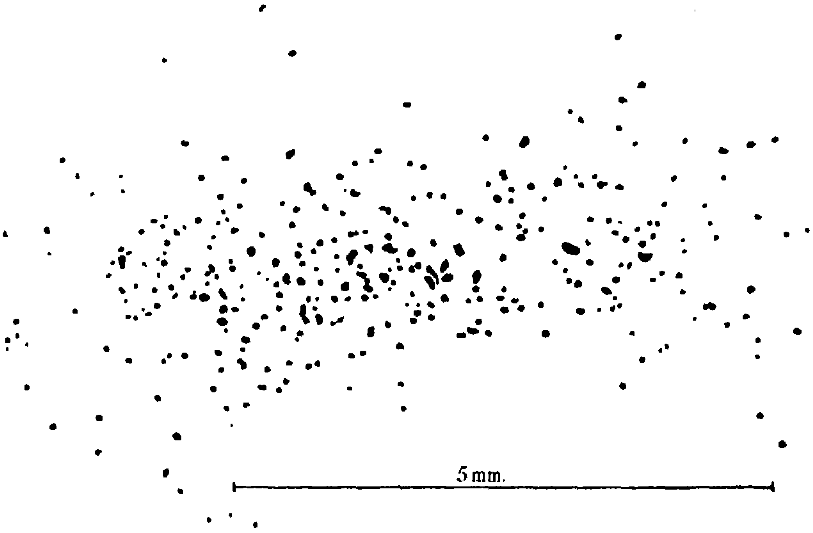


FIGURE 7. *Conocephalum conicum* (L.) Dum. Spray collected on a slide held horizontally 7 cm. above a discharging antheridium. The finer droplets contain about five spermatocytes.

The mechanism of the dehiscence of *M. domingensis* has been studied by Anderson (1931, p. 66). She observed the spermatocytes emerging from the antheridium in a long smoke-like column in the water.

We collected material of *M. polymorpha* in June and found that it behaves in the same way as *M. domingensis* but we also observed that when the drop was transferred to a glass slide the spermatocyte masses had disintegrated and the spermatocytes were lying separated from one another on the surface of the water. The sperms are liberated in a very few minutes. When only a small drop of water was used the spermatocyte masses did not completely disintegrate but the addition of water completed its disintegration. Nile blue sulphate and Sudan III gave positive indications of fat in the spermatocyte masses.

Jungermanniales—Anacrogynae

Pellia epiphylla L. A drop of water was placed on part of a thallus on which antheridia were present. The thallus was then placed in the sunshine. Dehiscence took place in a few minutes. A whitish mass appeared to rise to the surface. The thallus was then inverted on to a slide and the drop of water which adhered to the slide was examined under a microscope. Some of the spermatocyte mass was seen spreading over the surface.

Antheridia were pushed out of the cavities with a needle and placed in a drop of water on a slide. Natural dehiscence of the antheridium was not observed, but if an antheridium was pricked with a needle the contents came out and spread over the surface film.

There were a large number of spermatocytes on the surface of the water and some spermatozoids were swimming freely through the water.

The spermatocyte membrane was more distinct in *Pellia* than in the spermatocytes of the other Bryophytes examined.

Micro-chemical staining gave rather varied results. Ruthenium red and methylene blue gave positive reaction for mucilage. Sudan III and Sudan IV showed the presence of fat in the mass generally, but Nile blue sulphate reaction was more difficult to obtain but with some material the spermatocyte walls stained pale pink indicating the presence of fat.

Pellia fabbrowiana Raddi. Showalter (1926, p. 704) observed that the discharged 'antherozoids' of this hepatic float on the surface of water.

Aneura pinguis (L.) Dum. Showalter (1926, p. 694) has described how the ripe antheridium when covered with water produces a small irregular column, like a column of smoke, which rises to the surface of the water, becoming larger in diameter and less definite in outline. It may disperse before it is 1 mm. in length. He describes how the whitish mass became separated into what were evidently the spermatocytes which seemed to repel one another. Jerky movements were seen in the mass. The spermatocytes rose to the surface and floated for a few minutes. The sperms were liberated from the spermatocytes in from 2 to 20 min.

Showalter also discovered that when the surface of the thallus was wetted and brought in contact with a glass slide the antherozoids spread over the glass; he writes, 'this suggests the probability of a phenomenon of surface tension but our observations do not justify any final conclusion upon this question'.

Anthocerotales

Anthoceros laevis L. Only a small amount of *Anthoceros* with antheridia was available and a full investigation could not be carried out.

Natural dehiscence was not observed, but when antheridia were allowed to dehisce into a drop of water which was then transferred to a slide and examined microscopically, the spermatocytes could be seen lying in the surface film. The film was allowed to dry on to the slide and stained with gentian violet. The interesting fact was then observed that the spermatocytes were spaced with remarkable regularity over the surface of the water (figure 5, plate 14).

A positive reaction for fat was obtained with Sudan III, but no other tests were carried out.

CONCLUSIONS

Dehiscence of the antheridium

In a large number of species of mosses the dehiscence of the antheridium is preceded by a bursting inward of the cap cell and in some instances the adjoining cells at the apex of the antheridium. The pressure inside these cells is due to the swelling of the mucilaginous layers on the inner side of their outer cell walls. The contents of these burst cells are discharged on to the top of the spermatocytes.

The outer wall of the cap cell is then ruptured by the pressure exerted by the contents of the antheridium and the sperm mass is extruded as a sausage-shaped mass. The shape and size of the antheridium does not alter appreciably and as fast as the spermatocyte mass moves out a clear colourless fluid accumulates behind it at the base of the antheridium. As the spermatocyte mass expands slightly on emerging from the antheridium it cannot be losing water and the water in the fluid must enter through the cells at the base of the antheridium. It must be the hydrostatic pressure of the fluid which forces the spermatocyte mass out of the antheridium. The nature of this fluid and the mechanism of its entry into the cavity of the antheridium has not yet been determined.

Dispersal of the spermatocytes

The spreading apart of the spermatocytes on the water surface after the discharge of the antheridial contents is evidently a phenomenon of widespread occurrence in Bryophyta. With the exception of *Sphagnum* it has been observed in all the mosses so far investigated. In the liverworts it occurs in *Marchantia*, *Pellia*, *Aneura* and *Anthoceros* and we have some inconclusive evidence that it occurs in *Scapania*. It does not occur in *Conocephalum*.

It is significant that while fat may be detected in the sperm mass of all those species which exhibit the phenomena it cannot be detected in *Sphagnum* and *Conocephalum*. It is probable, therefore, that fat is responsible for lowering the surface tension and causing the spread of the spermatocytes on the surface. The extraordinary regularity in the distribution of the spermatocytes may be due, as Professor Thomas Alty has suggested to us, to the existence of an electrical charge on the spermatocyte causing repulsion. Experiments are being devised to test this suggestion.

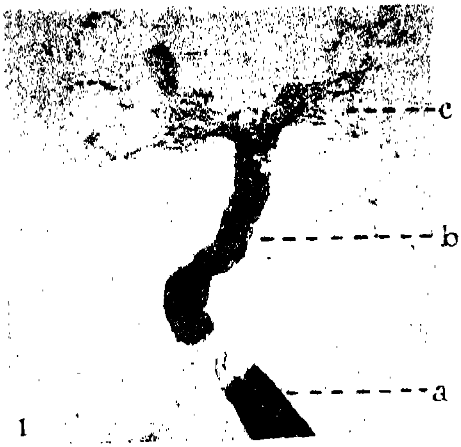
Biological significance of spermatocyte dispersal

In Hepaticae the main effect of this phenomenon is to carry the spermatocytes rapidly away from the antheridium. The speed of this movement is much greater than that of a free sperm. The spermatocytes rapidly cover the surface of the wet thallus, and some are likely to be brought very close to the archegonia on monoecious plants. When the sperm itself is liberated it has probably a very short distance to travel and is within range of chemotactic influence. It would appear, therefore, that the surface spreading of spermatocytes is as important as chemotaxy in ensuring fertilization. In *Marchantia* and probably some of the other Marchantiales it is possible that rain water splashed from the male plant bears a film of spermatocytes. It is obvious that the surfaces of plants are most likely to be wet and the antheridia discharging spermatocytes when it is raining. In other Marchantiales, e.g. *Conocephalum*, the violent discharge of spermatocytes in droplets in the form of a fine spray takes the place of surface spread and it is significant that in this type of mechanism no oil is present in the spermatocyte mass and no spreading of the spermatocytes by surface tension occurs.

In many Musci the spreading or cup-shaped form of the perichaetium suggests an adaptation for catching and holding water which is given a surface layer of spermatocytes. This layer may be removed by small insects or by rain but the surface layer is immediately replenished from the undispersed spermatocyte masses remaining. The fact that the antheridia in a perichaetium are usually at different stages of development suggests that over a period of two or three months, whenever water is present, a film of spermatocytes is available for dispersal.

It may be easily demonstrated that in comparison with a suspension, in which the particles are spread throughout the liquid, an arrangement in which the particles are concentrated in the surface layer allows of the removal of a far greater number of particles by an object dipped into the liquid and then withdrawn.

It cannot be said that the sporophytes of Bryophyta and Pteridophyta show much difference in their degree of adaptation to land conditions. It is evident, however, that the gametophytes of Bryophyta, in their method of dispersal of the gametes, are the much better adapted of the two groups to land conditions. In the majority of the Bryophytes use is made of the water surface and of insects for the transport of gametes, while in the Pteriodphyta the gametes are entirely aquatic in character.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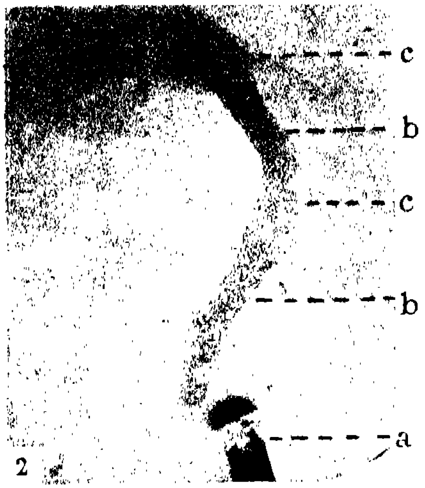
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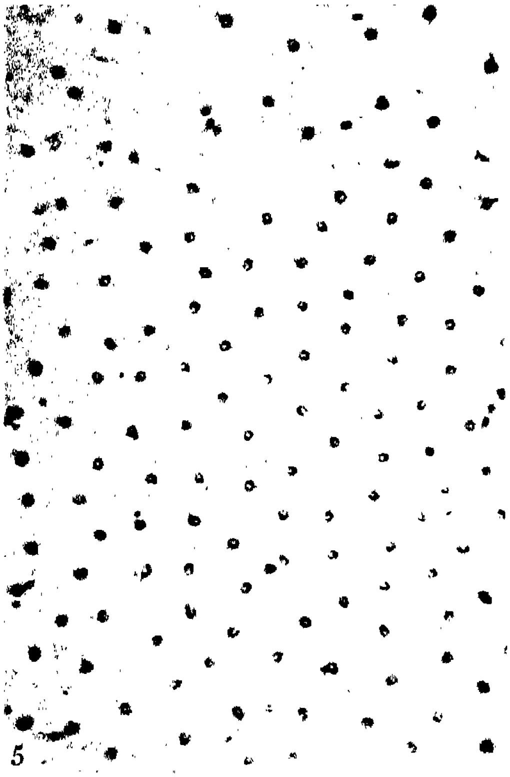
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DESCRIPTION OF PLATE 14

Mnium hornum L. Photographed from living material

FIGURE 1. Antheridium from which the mass of spermatocytes has been discharged. 45 × nat. size. *a*, apex of antheridium showing ruptured cap cell; *b*, spermatocyte mass below water surface; *c*, spermatocytes and mucilage spreading over the surface of the water.

FIGURE 2. Antheridium with discharged mass of spermatocytes. The latter has come into contact with the surface at two points *c*, *c*. 25 × nat. size. Lettering as for figure 1.

FIGURE 3. Spermatocytes in which the coiled body of the sperm is visible spreading over a water surface; stretched strings of mucilage may be seen between some of the cells. About 250 × nat. size.

Mnium punctatum L.

FIGURE 4. Part of a dispersed spermatocyte mass showing the cells still adhering to strands of mucilage. The preparation was allowed to dry on the slide and was then stained in gentian violet before the photograph was taken. 435 × nat. size.

Anthoceros laevis L.

FIGURE 5. Portion of a dispersed mass of spermatocytes allowed to dry on the slide and stained in gentian violet before the photograph was taken. The spermatocytes at the top of the photograph were near the edge of the expanding mass. 435 × nat. size.

Intra-uterine mortality of the wild rabbit, *Oryctolagus cuniculus* (L.)

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(*Communicated by D. M. S. Watson, F.R.S.—Received* 19 *December* 1941)

The paper deals with a sample of 185 pregnant rabbits obtained in Caernarvonshire between February and July 1941 inclusive, during the height of the breeding season. The intra-uterine mortality was estimated by comparison of the number of corpora lutea in the ovaries with the number of healthy embryos in the uteri. By grouping the data according to the stage of development of the embryos, it is shown that between 42 and 56 % of all litters are lost before the 20th day of gestation. This enormous mortality occurs chiefly on the 11th and 12th days of gestation, after implantation and when the placenta is already well established. Analysis of the mortality according to the initial size of litter shows that the proportion of litters showing loss increases with the initial size of the litter in the form of a geometrical progression. In almost every litter-size class a relatively large number of litters were observed in which all the embryos were reabsorbing. The mortality, whether represented by the percentage of animals showing loss or by the percentage of ova lost, also varies according to (*a*) whether the animals are, or are not, lactating, (*b*) the cleaned body weight of the mother, and (*c*) the month. These variations are, however, fully accounted for by variations in the frequencies of the initial size of litter.

INTRODUCTION

The reproductive processes of animals in the wild state often differ widely from those of the same species in captivity or domestication. It is therefore dangerous to apply knowledge of reproduction derived exclusively from laboratory animals to their wild progenitors. Our knowledge of the reproduction of the rabbit, although extensive, is derived mainly from domesticated breeds and very little is known of these processes in the wild animals. Consequently an investigation of the reproduction of wild rabbits has been undertaken in the hope that light might be thrown on the biology of the species which would be of assistance to those concerned with its control. This investigation was intended to be complementary to the work on the ecology and bionomics of the species proceeding at Oxford. In view of the unexpected nature of the results obtained concerning intra-uterine mortality, it was felt to be desirable to publish these at once rather than to hold them over until the investigation of other aspects of reproduction had been completed.

MATERIAL AND TECHNIQUE

The material consisted of a weekly sample of between twenty and forty marketable rabbits obtained in Caernarvonshire during the months of February to July 1941, inclusive. These were paunched by the trappers, care being taken to avoid damaging the reproductive organs, and were marketed after examination and removal of the reproductive organs. The samples were composed of rabbits fit for market, the trappers tending to exclude very small rabbits, unless they had a bunch the equivalent of an adult couple, and those that were very badly damaged. Every effort was made to ensure that no other selection was exercised. The material used during February, March and the first fortnight of April was shot on the Vaynol Estate, near Bangor. That used subsequently was trapped on the Lleyn peninsula in south Caernarvonshire. These two localities are some 16 miles apart. The cleaned body weight of all animals was recorded. Milk that could be expressed from the nipple was used as the criterion of lactation. Since milk is present on and after the 29th day of gestation in the glands of pregnant rabbits, which are not suckling, this criterion did not enable suckling and non-suckling animals near full-term to be distinguished. The corpora lutea in the ovaries and the uterine swellings, when visible in pregnant animals, were counted before removal of the reproductive organs. Little difficulty was experienced in accurately counting the number of corpora lutea during pregnancy, but it should be noted that if any error arose through failure to recognize a corpus luteum it would tend to reduce the apparent mortality. All embryos were counted as healthy unless they showed obvious and unmistakable signs of being in process of reabsorption. Thus all embryos that were suspected of being in early stages of reabsorption were classed as healthy. This stringent criterion of reabsorption tended to reduce the apparent mortality. The age of litters in which all the embryos were reabsorbing was determined, where possible, from the remains of the least autolysed embryo. Only two animals are included in the data in which reabsorption of all the embryos had proceeded too far to determine the stage at which the last died. It was found that the macroscopic appearance of the unopened uterine swelling was a good guide to the condition of the contained embryo. Swellings that were spindle-shaped rather than spherical and with a slightly wrinkled uterine surface, coloured by extravasation of blood in the tissues, often contained reabsorbing embryos. Those that were spherical, tumid and clear in appearance always contained healthy embryos. This distinction was especially apparent after fixation.

It was found to be possible, with practice, to recover without loss tubal ova or unattached uterine blastocysts by transfusion with saline. A pipette of suitable size, fitted with a rubber bulb, was employed and the ova or blastocysts were identified and recovered under a binocular dissecting microscope. The Fallopian tube was severed from the uterus and dissected away from the ovary, the mesentery being removed before transfusion. The canula was always inserted at the fimbriate end and care was taken not to puncture the tube at any point. The ova or blastocysts, when recovered, were fixed in Bouin's fluid. Early stages were subsequently attached to small blocks of testicular tissue by means of a drop of Meyers glycerine and albumen coagulated with absolute alcohol, and were serially sectioned. Blastocysts which had become attached and could not be removed from the uterus by transfusion always formed macroscopically visible swellings on the uterus.

Implanted embryos between 8 and 20 days old inclusive were aged by comparison with the figures in the normal table (Minot & Taylor 1905). Earlier stages were aged as shown in the following table 1.

TABLE 1

age	position	technique	characters
0 day stage	tubal	serial sectioned	up till and including 1st cleavage division
1 ,, ,,		,, ,,	2-cell to 16-cell stages
2 ,, ,,		,, ,,	more than 16 cells
3 ,, ,,		,, ,,	morulae or very early blastocysts
4 ,, ,,	uterine	whole specimens	small blastocysts
5 ,, ,,		,, ,,	medium blastocysts
6 ,, ,,		,, ,,	large blastocysts
7 ,, ,,		mounts of blastoderm	compared with figures of Minot & Taylor (1905)

Embryos more than 20 days old were aged by the mean weight of the embryos removed from the membranes, after fixation in Bouin's fluid and preservation in 70% alcohol. The following table 2 of weights derived graphically from embryonic growth curves of domestic breeds of rabbits was employed.

TABLE 2

age	mean weight g.	age	mean weight g.
21 day stage	3·7– 5·4	27 day stage	18·8–23·2
22 ,, ,,	5·4– 7·5	28 ,, ,,	23·2–29·0
23 ,, ,,	7·5– 9·8	29 ,, ,,	29·0–35·8
24 ,, ,,	9·8–12·4	30 ,, ,,	35·8–42·0
25 ,, ,,	12·4–15·3	31 ,, ,,	42·0–45·7
26 ,, ,,	15·3–18·8	32 ,, ,,	45·7 +

Since the Normentafeln, used as the basis of this system of ageing, refers to domestic breeds of rabbits in which gestation lasts approximately 32 days, it was convenient to divide the gestation period for the wild rabbit into a similar number of stages. Actually gestation in the wild animal is certainly shorter, probably 28–30 days as stated by Southern (1940). Therefore each daily stage actually represents an interval of 21 hr., not 24 hr. as in the domestic breeds on which the tables are based. Apart from the discrepancy arising from this cause it is probable that the method of estimating the age is accurate to ± 1 day, except at the end of gestation when the margin of error may be slightly greater.

OBSERVATIONS

Magnitude of mortality

The data presented in table 3 concern the incidence of intra-uterine mortality of embryos according to the stage of gestation. The mortality in utero can be represented, as in the table, either as the proportion of litters in which one or more ova have been lost or as the proportion of all ova ovulated that are lost. Ovulation is not spontaneous in the rabbit, but is consequent on copulation, so that the probability of ova being unfertilized is very small and can play only a very insignificant part in the loss, as is borne out by examination of serial sections of all the tubal ova recovered.

The mortality in utero is estimated from the difference between the number of ova ovulated, as shown by the number of corpora lutea, and the number of healthy embryos remaining. Since the corpora lutea persist throughout pregnancy irrespective of the death and disappearance of some of the embryos, it follows that the estimated mortality in comparable samples should increase at successive stages of gestation. The loss shown by each age group includes the loss in that sample at earlier stages of development. Therefore it follows from the data in table 3 and figure 1, that the samples in the successive age groups are not comparable and that the death of embryos on or before the 20th day must result in many instances in the loss of the whole litter and the cessation of pregnancy. The embryos must be reabsorbed rapidly and the animals, being then no longer pregnant, do not form part of the population represented by the samples of the later age groups. This is borne out by the fact that no animals more than 20 days pregnant were observed to have lost more than two ova each or to have less than two healthy embryos each remaining.

There are three methods by which the percentage of litters that are lost in this way can be estimated from the data. These are:

(1) Mortality is at a maximum in the 11–15 day age group, which consists of fifteen litters showing no loss of ova and thirty-two litters showing some loss. Those showing some loss included both litters in which all the embryos will be lost and litters in which some of the embryos will survive. The later age groups will contain only litters in which some or all of

the embryos survive. The age groups 21 days pregnant and over consist of forty-five litters showing no loss and seventeen litters showing some loss. Therefore the maximum number of litters showing some loss that may be expected to survive in the 11–15 day age group is: $\frac{17 \times 15}{45} = 5{\cdot}6$. It follows that a loss of 26·3 or 56 % of the litters is to be expected. This is a minimum estimate because if any embryos which died after the 15th day have been included, their inclusion will have decreased the estimate.

TABLE 3. MORTALITY IN UTERO, ACCORDING TO STAGE OF GESTATION

age groups days	total number of litters observed	number of litters showing loss	percentage of litters showing loss	total number of ova	total number of ova lost	percentage of ova lost
< 10	36	8	22·2	170	19	11·2
11–15	47	32	68·1	244	124	50·8
16–20	38	20	52·6	192	36	18·8
21–25	30	7	23·3	151	9	6·0
26 <	32	10	31·2	156	12	7·7
total	183	77	42·1	913	200	21·9

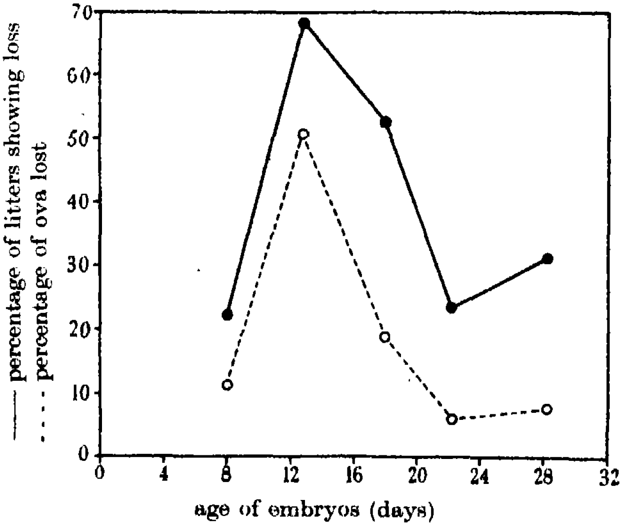

FIGURE 1

(2) The relative numbers of animals obtained in early and late stages of gestation provides a means of estimating the percentage of litters lost, assuming that the method by which they were obtained exercises no selection between those in different stages. During the first 10 days of gestation ninety-six animals were obtained compared to sixty-seven during the last 12 days, a difference that would be satisfied by a loss of 42 % of the litters.

(3) No animals which had lost more than two ova were obtained after the 18th day, but twenty-two, that is 47 %, of those in the 11–15 day age group had lost three or more ova. This method assumes that of the 11–15 day age group none of those litters in which only one or two ova have died will be lost; if they are, the percentage of litters lost will be greater than that estimated.

The fundamental assumptions on which the validity of each of these three methods depends are different in each case, yet the estimates they yield are not widely divergent. It must be concluded that at least 42–56 % of all litters are lost between the 10th and 20th days of gestation.

Stage of development at which maximum mortality occurs

The incidence of reabsorption of embryos appears to be highest during the 11th and 12th days of gestation. Of twenty-four animals 11 or 12 days pregnant, fifteen had three or more embryos reabsorbing and of these ten had all the embryos reabsorbing. No animals were obtained with three or more embryos reabsorbing before the 9th day or after the 18th day of gestation. No embryos actually in process of reabsorption were found after the 20th day. The data are given in table 4 and figure 2. It may be concluded that the loss shown by animals 20 days pregnant and over was sustained at an earlier period in gestation, and that the death of an embryo after the 20th day, if it occurs at all, is followed by abortion, not reabsorption. The small number of animals obtained with uteri in an immediately post-partum condition may have included some that had recently aborted advanced embryos and which would have been very difficult to distinguish from those that had given birth recently to living young.

Distribution of mortality according to initial size of litter

The relation between mortality and the initial size of litter, as determined from the number of corpora lutea in the ovaries, is shown in table 5. This table shows clearly that in almost every litter-size class a relatively large number of examples were observed in which all the embryos were reabsorbing. This corroborates the interpretation of the data presented in table 3, for if a large number of litters are lost between the 12th and 15th days of gestation, as is shown in table 4, it is to be expected that a number of examples would be observed in which all the embryos were in process of reabsorption.

It must be remembered that animals in which the whole litter is lost cease to be included in the sample once the embryos and corpora lutea have disappeared and they are no longer pregnant. The number of such animals must be considerable, since it has been shown that 42–56 % of the litters are lost by the 20th day of gestation. It may be suggested

TABLE 4. TIME OF REABSORPTION OF EMBRYOS

stage day	number of examples	number of examples containing reabsorbing embryos	number of embryos reabsorbing
9	12	1	3
10	6	1	6
11	10	8	29
12	14	9	40
13	8	2	3
14	7	5	14
15	8	5	16
16	12	3	7
17	5	2	4
18	6	3	4
19	3	2	3
20	12	3	5

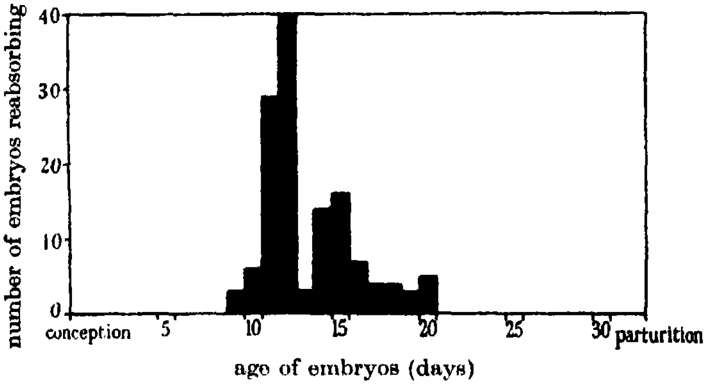

FIGURE 2

TABLE 5. MORTALITY ACCORDING TO INITIAL SIZE OF LITTER

initial size of litter	number of litters observed									total number of ova	
	total	0 ova lost	1 ovum lost	2 ova lost	3 ova lost	4 ova lost	5 ova lost	6 ova lost	7 ova lost	ovulated	lost
8	4	1	—	1	1	—	—	—	1	32	12
7	22	8	4	5	1	1	—	1	2	154	41
6	40	22	5	3	1	1	—	8	—	240	66
5	47	26	8	5	2	2	4	—	—	235	52
4	44	31	10	2	—	1	—	—	—	176	18
3	28	18	5	1	4	—	—	—	—	84	19
total	185	106	32	17	9	5	4	9	3	921	208

that table 5 is misleading, since it is admittedly biased by the exclusion of all those litters in which the reabsorption of the embryos was complete. It is instructive, therefore, to examine the relation between mortality and the initial size of litter for each age group separately. The data are given in table 6.

It will be observed that the proportion of litters showing loss of ova increases as the initial size of litter rises. The relation approximates to a geometrical progression where the probability that any litter of n ova will suffer no loss is

$$1 = p^n \quad \text{or} \quad \log 1 = n \log p. \tag{1}$$

The mean value of $p = 0{\cdot}892$ for the whole sample can be calculated from the values derived from each litter-size group, as in the left-hand section of table 7. This empirical formula is fitted to the data, using the mean value of $p = 0{\cdot}892$, in the right-hand sections of the table. The goodness of fit is tested by means of χ^2, calculated both from the litters showing loss and those showing no loss. Three degrees of freedom are lost, two since the expected frequencies have been calculated from the observed total and the mean value of p, and one by combining the litter-size classes of 7 and 8, leaving $n = 3$. P is found to be between 0·3 and 0·5, and therefore the empirical formula fits the data well. This formula provides a convenient means of correcting for the effect of differences in litter size when comparing the mortality in different fractions of the sample, as in the succeeding sections.

Influence of lactation on mortality

The maintenance of pregnancy during lactation is exceptional in domesticated rabbits, and the possibility that the very high mortality rate in utero observed in wild rabbits is associated with lactation suggests itself. The data are grouped in table 8 according to whether the animals were, or were not, lactating. Animals 29 days pregnant and over were excluded because the presence of milk in the glands does not necessarily indicate that they are already suckling. It can be seen that the mortality, as measured either by the percentage of litters showing loss or the percentage of ova lost, is distinctly higher in all the age groups in the lactating animals. It is necessary to determine whether this difference is due to lactation or whether it can be accounted for by a difference in the frequencies of litter sizes in the two classes. The frequencies of litter sizes in the two classes are given in table 9 together with the observed number of litters showing no loss. The expected distribution of litters showing no loss is calculated from equation (1), using the mean value of $p = 0{\cdot}892$ derived from the whole sample of litters. It can be seen that the observed and expected values agree very closely in each class, and it may be concluded that the difference between the proportion of animals showing loss in lactating and non-lactating animals is due to differences in the frequencies of litter sizes. Therefore the data provide no evidence that in the wild rabbit lactation has any appreciable effect on intra-uterine mortality.

TABLE 6. MORTALITY ACCORDING TO INITIAL SIZE OF LITTER IN EACH AGE GROUP

		number of litters observed									total number of ova	
age group days	initial size of litter	total	0 ova lost	1 ovum lost	2 ova lost	3 ova lost	4 ova lost	5 ova lost	6 ova lost	7 ova lost	ovulated	lost
> 10	8	1	1	—	—	—	—	—	—	—	8	—
	7	4	2	1	—	—	—	—	—	1	28	8
	6	5	3	1	1	—	—	—	—	—	30	3
	5	7	6	—	1	—	—	—	—	—	35	2
	4	12	11	1	—	—	—	—	—	—	48	1
	3	7	5	—	1	1	—	—	—	—	21	5
total		36	28	3	3	1	—	—	—	1	170	19
11–15	8	2	—	—	—	1	—	—	—	1	16	10
	7	5	1	1	1	—	—	—	1	1	35	16
	6	14	3	1	—	1	1	—	8	—	84	56
	5	13	4	2	1	1	2	3	—	—	65	30
	4	5	3	—	1	—	1	—	—	—	20	6
	3	8	4	3	—	1	—	—	—	—	24	6
total		47	15	7	3	4	4	3	9	2	244	124
16–20	8	—	—	—	—	—	—	—	—	—	—	—
	7	7	3	1	1	1	1	—	—	—	49	10
	6	6	2	2	2	—	—	—	—	—	36	6
	5	13	8	1	3	1	—	—	—	—	65	10
	4	6	2	3	1	—	—	—	—	—	24	5
	3	6	3	2	—	1	—	—	—	—	18	5
total		38	18	9	7	3	1	—	—	—	192	36
21–25	8	—	—	—	—	—	—	—	—	—	—	—
	7	4	1	1	2	—	—	—	—	—	28	5
	6	7	7	—	—	—	—	—	—	—	42	—
	5	7	4	3	—	—	—	—	—	—	35	3
	4	10	9	1	—	—	—	—	—	—	40	1
	3	2	2	—	—	—	—	—	—	—	6	—
total		30	23	5	2	—	—	—	—	—	151	9
26 <	8	1	—	—	1	—	—	—	—	—	8	2
	7	2	1	—	1	—	—	—	—	—	14	2
	6	8	7	1	—	—	—	—	—	—	48	1
	5	6	4	2	—	—	—	—	—	—	30	2
	4	11	6	5	—	—	—	—	—	—	44	5
	3	4	4	—	—	—	—	—	—	—	12	—
total		32	22	8	2	—	—	—	—	—	156	12

TABLE 7. NUMBER OF LITTERS SHOWING LOSS COMPARED WITH EXPECTATION

initial size of litter	total number of litters observed	proportion of litters observed showing no loss $=l$	$\sqrt[n]{l}=p$	$p\times$ total number of litters observed	number of litters observed showing no loss	number of litters expected showing no loss $=m$	divergence $=x$	x^2/m	number of litters observed showing loss	number of litters expected showing loss $=m$	divergence $=x$	x^2/m
8	4	0·250	0·841	3·364	9	11·5	−2·5	0·54	17	14·5	+2·5	0·43
7	22	0·364	0·867	19·074								
6	40	0·550	0·906	36·240	22	20·2	+1·8	0·16	18	19·8	−1·8	0·16
5	47	0·553	0·889	41·783	26	26·5	−0·5	0·01	21	20·5	+0·5	0·01
4	44	0·704	0·916	40·304	31	27·9	+3·1	0·34	13	16·1	−3·1	0·60
3	28	0·643	0·863	24·164	18	19·9	−1·9	0·18	10	8·1	+1·9	0·45
total	185			164·929	106	106·0	0·0	1·23	79	79·0	0·0	1·65

$$\bar{p}=\frac{164\cdot929}{185}=0\cdot892.\quad \chi^2=1\cdot23+1\cdot65=2\cdot88.\quad n=3.\quad P=0\cdot5-0\cdot3.$$

TABLE 8. MORTALITY IN UTERO ACCORDING TO AGE IN LACTATING AND NON-LACTATING ANIMALS

age group days	total number of litters observed	number of litters showing loss	percentage of litters showing loss	total number of ova	number of ova lost	percentage of ova lost
			non-lactating			
< 10	16	3	18·8	59	6	10·2
11–20	23	13	56·5	85	25	29·4
21 <	20	4	20·0	77	4	5·2
total	59	20	33·9	221	35	15·8
			lactating			
< 10	20	5	25·0	111	13	11·7
11–20	62	39	62·9	351	136	38·7
21 <	28	10	35·7	163	13	8·0
total	110	54	49·1	625	162	25·9

TABLE 9. NUMBER OF LITTERS SHOWING NO LOSS COMPARED WITH EXPECTATION IN LACTATING AND NON-LACTATING ANIMALS

	non-lactating			lactating		
initial size of litter	total number of litters observed	number of litters observed showing no loss	number of litters expected showing no loss	total number of litters observed	number of litters observed showing no loss	number of litters expected showing no loss
8	—	—	—	4	1	1·6
7	—	—	—	21	8	9·4
6	—	—	—	37	19	18·6
5	6	4	3·4	36	19	20·3
4	32	22	20·3	8	6	5·1
3	21	13	14·9	4	3	2·8
total	59	39	38·6	110	56	57·8

Seasonal variation of mortality

It can be seen from the data in table 10 that both the percentage of animals showing loss and the percentage of ova lost rise from a minimum in March to a maximum in May and then fall to a lower level in June and July. The monthly frequencies of litter sizes are given in the first section of the table. The observed frequencies of litters showing no loss are compared with the expected frequencies, calculated from equation (1) using the mean value of $p = 0{\cdot}892$ in the second section of the table. It can be seen that the observed and expected frequencies agree well.

Applying the χ^2 test, as in table 7, $\chi^2 = 1{\cdot}69$, $n = 3$ and $P = 0{\cdot}7$ to $0{\cdot}5$, it is found that there is no significant difference between them. The monthly

TABLE 10. SEASONAL VARIATION IN MORTALITY

	number of litters observed							number of litters showing no loss		number of ova		
month	total	litters of 3	litters of 4	litters of 5	litters of 6	litters of 7	litters of 8	ob-served	expected	total number of ova	number of ova lost	percentage of ova lost
February	18	9	9	—	—	—	—	12	12·1	63	12	19·0
March	49	12	24	10	3	—	—	33	30·9	200	30	15·0
April	55	4	5	20	20	5	1	32	30·0	295	64	21·7
May	37	—	1	9	12	12	3	16	18·4	229	74	32·3
June	24	3	4	7	5	5	—	12	13·4	125	26	20·8
July	2	—	1	1	—	—	—	1	1·2	9	2	22·2
total	185	28	44	47	40	22	4	106	106·0	921	208	22·6

TABLE 11. GEOGRAPHICAL OR SEASONAL VARIATION IN MORTALITY

	number of litters observed							number of litters showing no loss			number of ova		
season	total	litters of 3	litters of 4	litters of 5	litters of 6	litters of 7	litters of 8	ob-served	expected	divergence	total number of ova	number of ova lost	percentage of ova lost
February–16 April	96	23	34	22	12	4	1	63	58·5	+ 4·5	423	67	15·8
17 April–July	89	5	10	25	28	18	3	43	47·5	− 4·5	498	141	28·3
total	185	28	44	47	40	22	4	106	106·0	0·0	921	208	22·6

observed and expected percentages of animals showing loss are represented graphically in figure 3, which shows that the curves intersect in April. This period corresponds with a change in the locality from which the material was obtained. Those obtained on or before 16 April were from the Vaynol Estate in north Caernarvonshire, whereas those taken subsequently were from south Caernarvonshire, some 16 miles distant. These two localities therefore were isolated from each other so far as any possible movements of population were concerned. It is therefore necessary to compare the data derived from these two localities, the samples from each being nearly equal in size. They are given in table 11. It can be seen that when the data are grouped in this manner the observed number of animals showing no loss is greater in the first half of the season and less in the second half than the expectation calculated from equation (1) using the mean value of $p = 0{\cdot}892$. The deviations are in each case 4·5 so that the two samples, when corrected for differences in the frequencies of litter sizes, differ from each other by nine out of seventy-nine litters or 11·4 % of all litters. This difference, though not very great, is probably significant and may represent either a seasonal or a geographical variation in mortality.

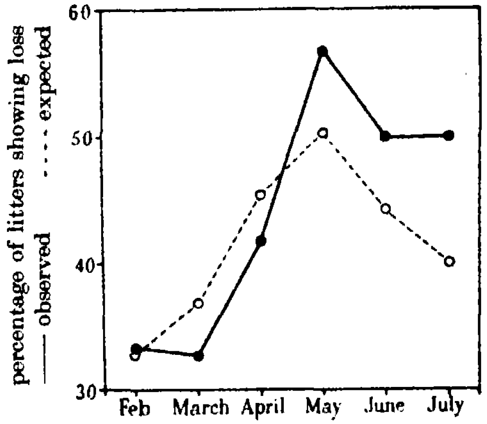

FIGURE 3

Variation in mortality according to the body weight of the mother

The data are grouped according to the cleaned body weight of the mother in 100 g. classes in table 12. It can be seen that the proportion of animals showing no loss decreases as body weight increases, reaching a minimum in the 1300–1399 g. class. The observed number of animals does not differ significantly from the expectation calculated from equation (1), using the mean value of $p = 0{\cdot}892$, and testing by means of χ^2. Entering the table with $\chi^2 = 5{\cdot}51$, $n = 3$, P is found to be between 0·2 and 0·1, and it may be

TABLE 12. VARIATION IN MORTALITY ACCORDING TO CLEANED BODY-WEIGHT

cleaned body-weight classes g.	number of litters observed							number of litters showing no loss		number of ova		
	total	litters of 3	litters of 4	litters of 5	litters of 6	litters of 7	litters of 8	ob-served	expected	total number of ova	number of ova lost	percentage of ova lost
1400 <	10	—	—	3	2	5	—	5	5·0	62	15	24·2
1300–1399	48	4	9	11	18	5	1	21	26·5	254	74	29·1
1200–1299	54	7	17	11	12	6	1	32	31·1	266	69	25·9
1100–1199	48	10	10	14	7	5	2	31	27·9	233	35	15·0
1000–1099	19	4	5	8	1	1	—	14	11·5	85	9	10·6
< 999	6	3	3	—	—	—	—	3	4·0	21	6	28·6
total	185	28	44	47	40	22	4	106	106·0	921	208	22·6

concluded that the variation in litter size frequencies accounts for the whole of the variation in the proportion of animals showing loss in the different body-weight classes.

CONCLUSIONS

Estimates of the amount of intra-uterine mortality in wild polytocous mammals are scarce, and those that have been made have been based on the discrepancy between the number of healthy embryos and the number of corpora lutea present. The samples available have, as a rule, been too small to permit of subdivision. Consequently each estimate has been based on the total mortality shown by the whole sample. Treating the rabbit data in this way as a single sample, a loss of 22 % of ova ovulated is apparent, which, although a high mortality rate for a wild animal, has been shown to be far below that which is actually experienced. The demonstration of the occurrence of a much higher mortality rate, and hence of the inadequacy of any estimate arrived at by treating the sample as a whole, depended on the fact that the embryos could be aged with considerable accuracy, owing to Minot & Taylor's (1905) normal table of development of domestic rabbits. Consequently the data could be grouped according to the stage of gestation and the loss of many whole litters on the 11th or 12th day was revealed. It is apparent that this method should be employed in the future and that estimates based on samples not grouped according to stage are open to serious criticism and may be entirely misleading.

The three methods employed of estimating the proportion of whole litters lost yield values between 42 and 56 %. In addition 6·8 % of the ova were lost in the remaining litters which survived the 20th day of gestation. Therefore it follows that between 45 and 58 % of all ova ovulated must have been lost. This is a very surprising conclusion, yet the technique was such that the experimental error should have tended to minimise the estimates, for any failure to recognize and count either a corpus luteum or an embryo in an early stage of reabsorption would have had this effect.

The discovery of this heavy rate of mortality in utero raises several problems requiring further investigation. It is important to determine whether this high rate of mortality is normal and relatively constant or whether it varies (*a*) from season to season, (*b*) in populations that are undisturbed as well as ones subject to intensive and prolonged destruction, such as those from which the data of this investigation were drawn, and (*c*) from locality to locality. Comparison of the samples from north and south Caernarvonshire suggest that there may be geographical variation. A single sample of sufficient size obtained by intensive effort during a limited period at the height of the breeding season would provide ideal material for comparison of annual and geographical variation in the incidence of intra-uterine mortality. Such information should be obtained without delay for it is obvious that a fluctuating mortality rate, liable to rise as high as that now recorded, could amply account for very large changes in density of population and might play an important part in the periodic changes in numbers of rabbits that are known to occur.

It is difficult for technical reasons to obtain information concerning the number of ova lost before the 7th day, when implantation occurs. The method of recovering ova and blastocysts by transfusion of the Fallopian tubes or uteri is reliable. Nevertheless, failure to recover by this means the full number ovulated is open to the criticism that it might be due either to mortality or to technical insufficiency. Consequently no attempt was made to estimate mortality by this means and only the Fallopian tube and uterus of the side on which the greatest number of ova had been ovulated was transfused as a rule. The only other means of obtaining the requisite data would have been by serially sectioning the Fallopian tubes and uteri, an immense labour with a large sample of animals the size of rabbits. The eight animals showing loss in the group 10 days pregnant or less all had attached blastocysts. They represented 22·2 % of animals showing loss and 11·2 % of ova lost. This loss must have included therefore the loss prior to implantation whether through ova failing to become fertilized or through death of the embryos. All the ova recovered by transfusion were sectioned and none were found to be unfertilized, but a few of them were too badly preserved to determine whether they were fertilized or not. It may be concluded that very few ova ovulated are lost through being unfertilized, doubtless because ovulation is dependent on copulation.

The maximum incidence of mortality on the 11th and 12th days of gestation is surprising for at this time the placental connection is well established and there is no obvious embryological reason why it should be a critical period. It might have been expected, a priori, that the incidence of mortality would have been highest around the 8th day, the time when implantation is effected. It is apparent from the data in figure 2 that the embryos and their membranes must be reabsorbed very quickly and that all traces of them must have been lost in a very few days after the death of the embryos. No reabsorbing embryos were obtained after the 20th day. Therefore either there is virtually no mortality after that stage or else all embryos that die after the 20th day are aborted. Abortion presumably involves the loss of the whole litter, as in other polytocous animals. Animals that had recently aborted a litter after the 20th day would be difficult to distinguish from post-partum animals and would probably have been classed as such. However, the number of animals obtained in this category was not large and could not have included many that had aborted.

The mortality, whether represented by the percentage of animals showing no loss or by the percentage of ova lost, varies according to (*a*) the initial size of the litters, (*b*) whether the animals are, or are not, lactating, (*c*) the cleaned body weight of the mother, and (*d*) the month. The proportion of animals showing no loss varies with the initial size of the litter in accordance with a simple equation (1). This equation provides a convenient means of correcting differences in the observed proportion of animals showing no loss arising from variation in the frequencies of litter sizes. For this purpose the mean value of the constant, p, for the whole sample was employed. Using this correction, it was found that the proportion of animals showing no loss does not vary significantly with the cleaned body weight of the mother, the month of the year nor according to whether or not the mother was suckling another litter. The observed variations are accounted for by variations in the frequencies of the initial sizes of litters.

The conclusion that the mortality rate in lactating and non-lactating wild rabbits does not differ significantly, if allowance is made for the distribution of litter sizes in the two groups, is in striking contrast to the observed effects of lactation on pregnancy in domestic rabbits. These are clearly stated by Hammond (Hammond & Marshall 1925), who says: 'While it is the rule in the rabbit that pregnancy does not occur during lactation there are two circumstances under which it is possible: (*a*) where only a small number of young are being suckled, and (*b*) in times of abundant nutrition suitable for breeding.' Hammond expresses the view that 'the lack of uterine nutrition as an effect of suckling causes the death of the blastocyst and its absorption'. The results of the present investigation do not support this view so far as the wild rabbit is concerned. On the contrary it must be concluded that the mortality observed was not due to any deficiency in the quantity of nutrient available for the developing embryos because (*a*) it takes place at a time when the demands of all the embryos in the litter can be only a fraction of those of a single foetus approaching full term, (*b*) it is not confined to large litters, and (*c*) it does not differ significantly in lactating and non-lactating animals when allowance has been made for the sizes of litters.

The causes of foetal atrophy are obscure. Hammond (1928) concluded that it was not due to bacterial action because the affected embryos are not necessarily confined to those that are lowermost in the uterus, where they would be most exposed to infection, but are scattered, often occurring between quite healthy embryos. He produced an inbred strain of rabbits in which much foetal atrophy occurred and he was able to show that, in this instance, it was due to a recessive mendelian factor, possibly a multiple factor. This factor operated through the mother on the foetuses in utero. It was therefore the genetic constitution of the mother and not that of the foetuses themselves which caused atrophy and the factor responsible was not a lethal. It is possible that the intra-uterine mortality in wild rabbits, recorded in this paper, is due to similar genetical causes, but that recorded by Hammond appears to take place at a later stage in the development of the embryos.

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